Molecular regulation of Sox2 expression during differentiation of chick embryonic stem cells

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'I, Ali Mahmoud Ghanem confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

Ali M Ghanem

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in The UCL Research Department of Cell and Developmental Biology, University College London under the supervision of Professor Claudio D Stern.

Dedication

To Syed Mohammad Hussein, my teacher, with all gratitude

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Thirty five springs have came and gone since I was born. Thirty summers since I first heard of what is a PhD! Walking by the side of my beloved cousin Hasan in the coastal Mediterranean city of Tartus he told me how those who set sail searching for the unknown are given a university degree called a doctorate! It was a fascinating picture he drew in my imagination – one of my long living memories. What Hasan did not tell me at the time is that to reach that destination a collective effort of so many people has to be woven together to make up the sail! Without such people, no journey would have even been possible let alone the arrival. I wish I could ever be able to list in here an exhaustive list of all people I am grateful for their help, support and encouragement which have been my sail of this journey. But I will try to put here those whose kind and generous actions will always speak louder than my humble words of acknowledgement.

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Abstract

The transcription factor Sox2 has a key role not only in maintaining stem state but also in specification of neural fate of embryonic cells. Multiple regulatory elements have been identified in the *Sox2* locus (Uchikawa et al, 2003). In the developing embryo, these regulatory elements are activated differentially in time and space. We studied the activity of 25 defined regulatory elements of the *Sox2* promoter in three different lines of chick ES cells. By transfection of plasmids encoding Enhanced Green Fluorescent Protein (EGFP) and the minimal promoter thymidine kinase (tk) coupled with individual *Sox2* regulatory elements we find that the *Sox2* enhancer N2 has the highest activity in proliferating chick cell lines compared with other enhancer regions. Under conditions that induce ES cells to differentiate into neurons the activity of the N2 enhancer increased along with an increase in levels of expression of *Sox2* mRNA. Further analysis of the N2 enhancer sequence identified two sub-regions with 176 and 73 base pairs (bp) which are highly conserved between chick, mouse and man. Functional studies performed with the tk-EGFP reporter plasmids under the control of five regulatory sequences containing the mouse N2 enhancer in its full length, its two sub-regions (176 and 73 bp) or sequences composed of the full length of the mouse N2 from which each of the two sub-regions 176 bp and 73 bp has been deleted confirmed that the two sub-regions of the N2 enhancer account for its activity in both proliferating cES cells as well as their induced neural differentiation state. These findings suggest that N2 core regulatory regions encode conserved instructions required to direct expression of *Sox2* both in embryonic stem cells induced to neural differentiation and in the neural plate of the embryo itself.

Contents

Acknowledgements 4
Abstract 5

CHAPTER 1. INTRODUCTION 12

1.1. Stem Cells 12

1.2. The chick embryo model and avian ES cells 15
1.2.1. Early development of the chick embryo 16
1.2.2. Chick embryonic stem cells 19

1.3. Molecular regulation of self-renewal and pluripotency 24
1.3.1. LIF pathway 24
1.3.2. Oct3/4 and its chick homologue POUV 25
1.3.3. Nanog 25
1.3.4. Sox2 27
1.3.5. iPS cells 28

1.4. Molecular mechanisms of neural specification 29
1.4.1. Neural Induction 29
1.4.2. Induction of neural differentiation in cultured ES cells 33

1.5. Sox2 and the SoxB1 family in neural development. 36

1.6. Aims of this thesis 39

CHAPTER 2. METHODS 42

2.1. Preparation of culture dishes and feeder cells 42

2.2. Preparation of conditioned medium 42

2.3. Generation of cES Cells from avian blastodermal cells 43

2.4. Maintenance of cES cells and cell lines 44

2.5. Reporter constructs for Sox2 enhancers 47

2.6. Transfection with LIPOFECTAMINE™2000 51

2.7. Cell counting and basic statistical analysis methods 57

2.8. In situ hybridization (ISH) of chick embryonic stem cells (cESCs). 58

2.8.1. Transcription of DIG-riboprobe 58

2.8.2. Preparation of cESCs for ISH 58

2.9. Immunohistochemistry and β-gal staining of chick embryonic stem cells (cESCs). 60

2.9.1. β-Gal staining 60

2.9.2. Immunohistochemistry and staining with DAPI 60

2.9.3. Antibodies 61

CHAPTER 3. IDENTIFICATION OF ENHANCERS RESPONSIBLE FOR SOX2 EXPRESSION IN CHICK ES CELLS 63

3.1. Introduction 63

3.2. Methods 64

3.3. Results 65

3.3.1. Activity of Sox2 conserved sequence blocks in cES cell lines and STO feeder cells 65

3.3.2. Statistical analysis of activity of Sox2 conserved sequence blocks 70

3.4. Discussion 76

CHAPTER 4. METHODS FOR INDUCING NEURAL DIFFERENTIATION IN CHICK ES CELLS 80

4.1. Introduction 80

4.2. Methods 83

4.2.1. Proliferating cES cells expansion 83
4.2.2. Development of embryoid bodies in vitro 83
4.2.3. In Vitro Differentiation on a monolayer 83

4.3. Results 84
4.3.1. Embryoid body-based protocol 84
4.3.2. Monolayer culture in defined medium 95
4.3.3. Expression of molecular markers in cES cells 106

4.4. Discussion 112

CHAPTER 5. GENE EXPRESSION PROFILE AND ACTIVITY OF THE N2 ENHANCER DURING INDUCED NEURAL DIFFERENTIATION OF CES CELLS 114

5.1. Introduction 114

5.2. Methods 116
5.2.1. Gene expression in cES cells 116
5.2.2. Induction of differentiation of cESCs 120
5.2.3. Plasmid design 120
5.2.4. Statistical analysis 123

5.3. Results 124
5.3.1. Patterns of gene expression during induced neural differentiation 124
5.3.2. Activity of the N2 enhancer and its core subregions during induced neural differentiation 138
5.3.3. Sox2 mRNA expression correlates N2 activity during induced neural differentiation 151

5.4. Discussion 156
5.4.1. Time course of changes in gene expression during induced neural differentiation of cES cells 156
5.4.2. Activity of the N2 enhancer and its sub-regions in cES cells 156

REFERENCES 168
APPENDIX 1 198

List of Figures

FIGURE 2.1: MORPHOLOGY OF CES CELLS 46
FIGURE 2.2: PLASMIDS USED FOR TESTING THE ACTIVITY OF SOX2 ENHANCERS IN CES CELLS 49
FIGURE 2.3: OPTIMIZING LIPOTRANSFECTION OF CES CELLS 54
FIGURE 2.4: LIPOTRANSFECTION OF CES CELLS - EFFICIENCY VS SURVIVAL 56
FIGURE 3.1: FUNCTIONAL ANALYSIS OF SOX2 REGULATORY BLOCKS IN PROLIFERATING CHICK EMBRYONIC STEM CELLS. 68
FIGURE 3.2: DIFFERENCES IN ACTIVITY OF DIFFERENT PLASMIDS IN DIFFERENT CESC LINES (9N2, 403, 30) AND STO FEEDER CELLS 71
FIGURE 3.3: DIFFERENCES IN THE ACTIVITY OF PLASMIDS BETWEEN STO AND CESC LINES. 75
FIGURE 4.1: EMBRYOID BODIES (EB) – LIKE AGGREGATES OF CES CELLS 86
FIGURE 4.2: CHANGES IN CELL MORPHOLOGY FOLLOWING INDUCED DIFFERENTIATION OF CES CELLS 90
FIGURE 4.3: NEURAL MARKER EXPRESSION IN CES CELLS FROM EMBRYOID BODIES . 92
FIGURE 4.4: QUANTIFICATION OF CES CELL DIFFERENTIATION FROM EMBRYOID BODIES 94
FIGURE 4.5: NEURAL MARKER EXPRESSION BY CES CELLS CULTURED IN N2B27 MEDIUM AS AN ADHERENT MONOLAYER (A) 98
FIGURE 4.6: NEURAL MARKER EXPRESSION BY CES CELLS CULTURED IN N2B27 MEDIUM AS AN ADHERENT MONOLAYER (B) 100
FIGURE 4.7: VARIOUS ALTERATIONS OF MONOLAYER BASED PROTOCOL INDUCED NEURAL DIFFERENTIATION OF CES CELLS. 102
FIGURE 4.8: NEURITE FORMATION OF CES CELLS FOLLOWING MONOLAYER N2B27 MEDIUM INDUCED DIFFERENTIATION PROTOCOL. 105
FIGURE 4.9: EXPRESSION OF NEURONAL MOLECULAR MARKERS IN N2B27 INDUCED CES CELLS 108
FIGURE 4.10: EXPRESSION OF NEURONAL MOLECULAR MARKERS IN N2B27 INDUCED CES CELLS (QUANTIFICATION) 110
FIGURE 5.1: FURTHER DISSECTION OF THE SEQUENCE OF THE N2 ENHANCER REVEALS 2 ESSENTIAL HIGHLY CONSERVED CORE SUBREGIONS 122

FIGURE 5.2: GENE EXPRESSION PROFILE OF THE 9N2 CES CELL LINE IN THE PROLIFERATIVE 'STEM' STATE 126
FIGURE 5.3: SOX2 EXPRESSION DURING INDUCED NEURAL DIFFERENTIATION OF CES CELLS. 131
FIGURE 5.4: CHANGES OF GENE EXPRESSIONS PROFILES OF CES CELLS 'STEM' STATE MARKERS DURING INDUCED NEURAL DIFFERENTIATION 133
FIGURE 5.5: CHANGES OF GENE EXPRESSIONS PROFILES OF CES CELLS 'PRE-NEURAL' & 'NEURAL' STATE MARKERS DURING INDUCED NEURAL DIFFERENTIATION. 135
FIGURE 5.6: EXPRESSION OF MESODERMAL (CBRA), ENDODERMAL (CSOX17) AND ECTODERMAL (BERT) MARKERS IN CES CELLS DURING INDUCED NEURAL DIFFERENTIATION. 137
FIGURE 5.7: ACTIVITY OF THE N2 ENHANCER AND ITS SUBREGIONS IN PROLIFRATING CES CELLS 142
FIGURE 5.8: ACTIVITY OF THE N2 ENHANCER AND ITS SUBREGIONS DURING INDUCED DIFFERENTIATION OF CES CELLS 147
FIGURE 5.9: ACTIVITY OF THE N2 ENHANCER AND ITS SUBREGIONS DURING INDUCED DIFFERENTIATION OF CES CELLS 148
FIGURE 5.10: PATTERNS OF ACTIVITY OF THE N2 ENHANCER AND ITS SUBREGIONS DURING A 10 DAYS TIME COURSE OF INDUCED NEURAL DIFFERENTIATION OF CES CELLS 150
FIGURE 5.11: COMPARISON BETWEEN DYNAMIC CHANGES IN SOX2 MRNA EXPRESSION AND THE ACTIVITY OF THE N2 ENHANCER DURING INDUCED NEURAL DIFFERENTIATION OF CES CELLS 153
FIGURE 5.12: DYNAMIC CHANGES IN SOX2 MRNA EXPRESSION CORRELATES WITH THE ACTIVITY OF THE N2 ENHANCER DURING INDUCED NEURAL DIFFERENTIATION OF CES CELLS 155
FIGURE 30 HYPOTHESIS DIAGRAM 167
FIGURE APPX.1: BIOINFORMATICS ANALYSIS OF THE N2 ENHANCER AND IDENTIFICATION OF CONSERVED TRANSCRIPTION FACTORS BINDING SITES (TFBS) 201
FIGURE APPX.2: SEQUENCE ANALYSIS OF THE N2 ENHANCER AND LOCALIZATION OF CONSERVED TRANSCRIPTION FACTORS BINDING SITES (TFBS) 202

List of Tables

TABLE 1.1: CHARACTERISTICS OF ES CELLS IN MOUSE, HUMAN AND CHICK MODELS . 23
TABLE 2.1: CONSERVED SEQUENCES OF SOX2 ACROSS AMNIOTE SPECIES 50
TABLE 2.2: SCALING OF TRANSFECTION VOLUMES WITH LIPOFECTAMINE TM 2000 FOR DIFFERENT SIZES OF WELLS 52
TABLE 2.3: COMPOSITION OF THE HYBRIDIZATION SOLUTION 59
TABLE 2.4: ANTIBODIES DESCRIPTION 62
TABLE 3.1: THE ACTIVITY OF THE IDENTIFIED ENHANCERS OF SOX2 GENE IN CHICK EMBRYONIC STEM CELLS 66
TABLE 4.1: REPORTS ON IN VITRO INDUCED NEURAL DIFFERENTIATION OF EMBRYONIC STEM CELLS IN MOUSE AND CHICK 82
TABLE 4.2: DIFFERENTIATION OF CES CELLS WITH AN EB BASED PROTOCOL. 88
TABLE 4.3: DIFFERENTIATION OF CES CELLS IN DEFINED MEDIA 103
TABLE 4.4: EXPRESSION OF NEURONAL (3A10 & TUJ-1) MOLECULAR MARKERS IN N2B27 INDUCED CES CELLS. 111
TABLE 5.1: HIERARCHY OF EXPRESSION OF TEN GENES INVOLVED IN REGULATION OF THE STEM STATE AND LINAGE COMMITMENT IN THE DEVELOPING CHICK EMBRYO. 118
TABLE 5.2: PROBES OF GENE MARKERS USED TO STUDY GENE EXPRESSION PROFILE IN DIFFERENTIATING CES CELLS 119
TABLE 5.3: EXPRESSION PROFILES OF 10 MARKER GENES DURING INDUCED NEURAL DIFFERENTIATION OF CES CELLS 129
TABLE 5.4: ANALYSIS OF THE ACTIVITY OF THE N2 ENHANCER AND ITS CORE SUBREGIONS DURING INDUCED NEURAL DIFFERENTIATION 143
TABLE 5.5: ANALYSIS OF VARIANCE IN THE ACTIVITY OF THE N2 ENHANCER AND SUBREGIONS IN CES CELLS 144
TABLE 5.6: PAIRED ANALYSIS OF ACTIVITY OF THE MOUSE N2 ENHANCER AND SUBREGIONS IN C ES CELLS. 145
TABLE APPX. 1: CONSERVED "HIGHLY SPECIFIC" TRANSCRIPTION FACTOR BINDING SITES (TFBS) IDENTIFIED IN THE SEQUENCE OF *SOX2* N2 ENHANCER USING MULAN SOFTWARE (LOOTS AND OVCHARENKO, 2007). 200

Chapter 1. Introduction

1.1. Stem Cells

Stem cells are defined as cells with the ability to self-renew, perhaps indefinitely, without becoming malignant or aneuploid (Atala, 2005;Constantinescu, 2003; Gardner and Brook, 1997; Loeffler and Roeder, 2002; Smith, 2001). In some instances, but not necessarily, stem cells may be pluripotent (ie. able to give rise to many cell types) or even totipotent (any cell type including the germ line). Endogenous stem cells with demonstrated self-renewing capacity have been found in many adult tissues, especially those that are subjected to abrasion or other cell loss and therefore require the cell population to be replenished throughout life. Well-studied examples include the basal layer of the skin (Watt, 1988; Watt, 1998), intestinal crypts (Potten, 1991; Potten and Loeffler, 1990), bone marrow and other haematopoietic precursor sites (Antica et al., 1994; Graham and Wright, 1997; Loutit, 1968) and the olfactory epithelium (Calof et al., 1998; Murray and Calof, 1999).

The term “stem cell” was first coined as long ago as 1908 by Alexander Maximov, who first proposed the concept of self-renewing progenitor cells to account for the generation of blood cells throughout life. However it was not until the 1960s that the concept became more firmly established through further studies of self-renewing cell populations both in the haematopoietic system, notably by Till and McCulloch (McCulloch and Till, 1964; Till and McCulloch, 1963) and in the skin and hair follicles, by C S Potten and others (Potten et al., 1979; Potten, 2004; Potten and Morris, 1988)

A further turning point came from studies on teratocarcinoma cells, which could be cultured indefinitely and cells retained their undifferentiated state. These cells were called embryonic carcinoma cells (ECCs) (Martin and Evans, 1975). This paved the way to the production of the first embryonic stem (ES) cells, derived from the inner cell mass of mouse blastocysts before

implantation (Evans and Kaufman, 1981; Martin, 1981). Eventually, ES cells were generated from human embryos (Shamblott et al., 1998; Thomson et al., 1998). Just before the turn of the century, ES-cell like cells were successfully generated from several other animal species such as the pig (Wheeler, 1994), rhesus monkey (Thomson et al., 1995) and chicken (Pain et al., 1996). In anamniotes, the establishment of cell lines with some ES-cell like characteristics has been described for zebrafish (Sun et al., 1995) but a demonstration that these are true ES cells or even pluripotent has yet to be provided.

Four essential features have been proposed to define Embryonic Stem (ES) cells. First, the key characteristic of all stem cells: they have the capacity, to self-renew, perhaps indefinitely, without change of their genotype or their phenotype. Second, ES cells are pluripotent (or even totipotent): they have the potential to differentiate into any somatic cell lineage, including derivatives of any of the three embryonic germ layers (ectoderm, mesoderm and endoderm). Third, in addition to their somatic pluripotency, true ES cells can contribute to germ cells: when transplanted into blastocyst-stage embryos they are able to contribute to every tissue type including the germ-line and can therefore give rise to a new individual. This property has been exploited in the last few decades as an effective way to construct transgenic mice, since gene manipulations can be done using homologous recombination in ES cells which can then be used to generate germ line chimaeras and generate transgenic animals (Gordon et al., 1980; Gordon and Ruddle, 1981; Ruddle, 1981; Shows and Sakaguchi, 1980). Finally, ES cells are able to form teratocarcinomas (undifferentiated malignant tumours composed of tissue resembling normal derivatives of all three germ layers - their name is derived from the Greek word “terato”, meaning monster) when they are introduced into adult tissue (Andrews et al., 2001; Matsui et al., 1992).

These characteristics distinguish ES cells from other cells with the capacity to self-renew and differentiate into multiple cell lineages with role in tissue repair and regeneration such as the haematopoietic system (Barnes and Loutit, 1967; Loh et al., 2009) and skin (Watt, 1998).

ES cells have attracted a great deal of interest by the public as well as in biomedical research domains. On one hand, their ability to self-renew and differentiate into many different cell types has raised hope for using them as tools for tissue regeneration and repair. On the other hand, their ability to integrate into host blastocysts and form chimaeras provides a powerful tool for studying developmental processes and gene function (Beddington and Robertson, 1989; Shows and Sakaguchi, 1980).

Over the last three decades, research on the biological properties of ES cells themselves has uncovered many mechanisms governing cells self-renewal, commitment and differentiation. Factors maintaining pluripotency have been identified, including the cytokine leukaemia inhibitory factor (LIF) (Niwa et al., 1998; Smith and Hooper, 1987) and intracellular proteins such as p21 and p53 (cell cycle regulators) (Aladjem et al., 1998; Savatier et al., 1996). More recently, it was discovered that transfection of just four transcription factors (Sox2, Klf4, Myc and Oct3/4) is sufficient to cause somatic, differentiated cells to re-enter the cell cycle and to become pluripotent. Cells so treated are known as induced Pluripotent Stem cells (iPS cells) (Takahashi and Yamanaka, 2006; Yamanaka, 2008) (see also below, iPS Cells).

These discoveries, along with renewed intensity of investigation into the molecular mechanisms governing cell and tissue replacement, ushered the birth of a whole new area, regenerative medicine. Examples of possible future applications include replacement of damaged cardiomyocytes following myocardial infarction (Nelson et al., 2009; Zhang et al., 2005), of dopaminergic neurons in Parkinson's disease (Chiba et al., 2008), of nephrons in end stage renal failure (Aboushwareb and Atala, 2008; Gupta et al., 2006; Koh and Atala, 2004; Perin et al., 2010)or of beta-islands cells in diabetes (Chang et al., 2007; Maehr et al., 2009; Zhao et al., 2006), to name just a few.

Understanding the processes behind cell fate decisions and lineage specification will be essential to direct differentiation of pluripotent cells into the required cell types to replace cells lost in disease or injury (Atala, 2009a;

Atala, 2009b; Smith, 2001). Although still limited, this approach has already started to benefit individual patients who have received stem cells or engineered tissues to reconstruct malformed or damaged structures (Macchiarini et al., 2008).

1.2. The chick embryo model and avian ES cells

During embryo development, all adult tissues arise from a single cell: the fertilized egg. Soon after fertilization this mother cell undergoes multiple cell divisions to generate daughter cells which progressively become specified to different fates. In amniotes, embryonic cells remain multipotent (or even totipotent) for several divisions before starting to become committed to sub-sets of fates. Understanding how the embryo allocates cell fates is crucial if we are to be able to control the differentiation of cultured ES cells to generate the desired cell types for clinical and other applications. Of particular interest are the mechanisms responsible for cells becoming specified to neural fates. The chick embryo has for a long time been a powerful model to study developmental processes including neural induction – how cells choose between neural and other fates (Stern, 2004b; Stern, 2005a; Stern, 2006). The early neural plate of vertebrates, from where the entire CNS arises, is initially defined by its expression of transcription factors of the SoxB1 class, including Sox2 (Collignon et al., 1996; Rex et al., 1994; Rex et al., 1997b; Uwanogho et al., 1995), which is one of the transcription factors identified as key regulators of pluripotency (see above). This project takes advantage of chick embryonic stem (cES) cells to investigate the molecular mechanisms that regulate Sox2 expression in pluripotent stem cells and in early neural cells.

The following sections briefly introduce the chick model, its early development and cES cells that can be derived from early chick embryos. Then, it reviews the mechanisms regulating multipotency and neural induction focusing on SoxB1 family of transcription factors.

1.2.1. Early development of the chick embryo

After fertilization, which is polyspermic, the chicken embryo develops in utero for about 20 hours before the egg is laid. Cleavage is meroblastic, or unequal, causing cells at the periphery of a cleaving disk to be open to the surrounding egg yolk (reviewed in (Stern, 2004a)). By the time of laying (stage X of fourteen pre-primitive streak developmental stages [Roman numerals I-XIV] defined by (Eyal-Giladi and Kochav, 1976) the embryo contains 20,000-50,000 cells (Spratt and Haas, 1960). They are arranged as a flat disk of about 3mm diameter, covered by an acellular vitelline membrane and separated from the yolk by the subgerminal cavity see (Stern, 2004a).

Two areas can be distinguished in the embryo at this stage: a more translucent inner region, the *area pellucida* and an opaque, thicker outer ring, the *area opaca*. All embryonic tissues arise from the *area pellucid*a which could therefore be considered to be equivalent to the mammalian inner cell mass. The outer *area opaca* gives rise only to extraembryonic tissues and is more similar to the trophectoderm of rodent embryos.

At stage X, the *area pellucida* is composed of a continuous layers of cells, the epiblast, an epithelial sheet that extends over both area pellucida and area opaca. The epiblast is underlain ventrally by loose “islands” of about 5-20 large, yolky cells: the hypoblast. Only the epiblast contributes cells to the three germ layers of the embryo (Stern, 2004a).

Soon after laying, the islands of hypoblast cells spread out to form a layer, expanding in a posterior to anterior direction to form the hypoblast layer proper. This layer does not contribute cells to the embryo but has several important functions in development including: directing cell movements of the overlying epiblast (Foley et al., 2000; Voiculescu et al., 2007; Waddington, 1932), preventing premature primitive streak formation while the axis relocates to the midline (Bertocchini and Stern, 2002), induction of the earliest steps of neural induction mainly through its secretion of FGF (Albazerchi and Stern,

2007; Foley et al., 2000; Streit et al., 2000) and as a vehicle to transport primordial germ cells to the germinal crescent (Ginsburg et al., 1989; Stern, 2004a). Stages X-XIII mark different stages in the expansion of the hypoblast layer, with stage XIII corresponding to the formation of a layer that fully covers the *area pellucida* (Eyal-Giladi and Kochav, 1976).

Immediately thereafter, a new cell type arises from deep cells at the posterior margin of the *area opaca/pellucida* border: the endoblast (Bertocchini and Stern, 2002; Stern, 1990) at stage XIV. This tissue displaces the hypoblast anteriorly. It has been shown that the hypoblast prevents premature primitive streak formation because it produces Cerberus, an inhibitor of Nodal, and removal of the hypoblast (and thereby of this inhibitor) by the expanding endoblast cells at the posterior end of the embryo releases Nodal signalling which initiates primitive streak formation from the overlying epiblast (Bertocchini and Stern, 2002).

From the time of appearance of the primitive streak a different staging system is used, described by Hamburger and Hamilton (1951) (HH), denoted in Arabic numerals. Stage 2 marks the appearance of a short, triangular primitive streak (the original HH Stage 1 has been replaced by the fourteen stages of (Eyal-Giladi and Kochav, 1976) [Roman numerals]).

Stages 2-4 of (Hamburger and Hamilton, 1951) correspond to the period known as gastrulation, during which the three definitive germ layers are laid down. The primitive streak is a midline thickening of the epiblast through which cells ingress and colonise the deeper parts of the embryo. A middle layer arises, and some of its cells migrate deeper still to colonise the hypoblast/endoblast layer, which eventually becomes displaced to the periphery of the embryo. The new deep layer is the definitive endoderm which will give rise to the lining of the gut and contribute to associated organs (Bellairs, 1953a; Bellairs, 1953b; Kimura et al., 2006; Sanders et al., 1978; Stern, 1990; Stern, 2004a; Stern and Canning, 1990; Stern and Ireland, 1981).

The cells remaining in the middle layer will become the mesoderm, subdivided into axial (prechordal and notochord regions), paraxial (including prospective somites), intermediate (Wolffian duct and mesonephric kidney), cardiac and lateral plate mesoderm. The latter will eventually give rise to the vasculature, blood islands, coelomic lining and limb mesenchyme and contribute cells to many internal organs; see (Bellairs and Osmond, 2005).

At HH Stage 4 the primitive streak has reached its full length and ingression stops through its most anterior part (Gallera, 1971;Sheng et al., 2003). The anterior tip of the primitive streak at this stage appears as a bulbous thickening, known as Hensen's node (Hensen, 1876). This is an important structure because it has the unique ability to induce a complete nervous system from epiblast cells when transplanted to an ectopic site; see (Stern, 2004a;Waddington, 1932). Immediately thereafter (HH Stage 5) the notochord starts to emerge as a rod extending anteriorly from the deep part of Hensen's node. Simultaneously the paraxial mesoderm emerges from the node and immediately posterior regions of the streak, and colonises the sides of the notochord in the middle layer (Psychoyos and Stern, 1996; Selleck and Stern, 1991). Both notochord and paraxial mesoderm subsequently elongate caudally as the primitive streak regresses, eventually to form the tail bud (Bellairs and Osmond, 2005)

Current evidence suggests that commitment of cells to a neural fate occurs between stages 4 and 5. The epiblast above and on either side of the portion of the notochord ("head process") that arose between these stages starts to become a more columnar, thickened epithelium, corresponding to the future cephalic neural plate. At stage 5, the position of the node corresponds to the level of the future otic vesicle (rhombomeres 5-6 of the hindbrain). The nervous system caudal to this level arises from two very small, paranodal regions of the Stage 5 epiblast (Diez Del Corral et al., 2003; Storey et al., 1995; Uchikawa et al., 2003).

From laying until immediately before the appearance of the primitive streak, the blastoderm has remarkable regulative ability. If a blastodisc is cut into fragments (halves, quarters or even eight pie-shaped pieces), each piece can generate a complete embryonic axis (Lutz, 1949; Spratt and Haas, 1960). We do not yet know to what extent individual cells of the embryo at this stage are multipotent or whether the embryo is a mosaic of cells of different prospective fates. However these findings reveal that the normal embryo contains mechanisms to generate pattern from a population of cells with multiple potentials, and also that during normal development embryos must possess mechanisms that suppress the formation of supernumerary axes (twins) other than at the posterior part of the *area pellucida*; see (Bertocchini et al., 2004; Spratt and Haas, 1960). This remarkable level of regulation is unique to amniote embryos. Because polarity of anamniote embryos is determined by localisation of maternal components during early cleavage stages and because of the absence of zygotic gene expression during the first 11 or so cell divisions, amphibian embryos lose their ability to regulate if cut after the third or so cell division (Stern, 2004a).

1.2.2. Chick embryonic stem cells

Chimera formation and germ line transmission was achieved by injection of chick stage X blastodermal cells into recipient embryos (Marzullo, 1970; Petitte et al., 1990).These findings prepared the way for the generation of cell lines from chick blastoderm cells that share many of the characteristics of mammalian ES or Epiblast Stem cells (Pain et al., 1996; Petitte et al., 2004; Van de Lavoir and Mather-Love, 2006). These cell lines can be maintained indefinitely. However although cES cell lines have the ability to contribute to most if not all somatic cell lineages, they do not appear to be able to contribute to the germ line either in vitro or in chimaeras (Lavial and Pain, 2010). Therefore cES cells are more akin to mouse Epiblast Stem Cells (Epi Stem cells) than to true mammalian Embryonic Stem cells (ES cells).

1.2.2.1. Isolation and culture conditions

Chick ES cells are derived form stage X blastodermal cells by culturing them on feeder layers of irradiated embryonic fibroblasts or other cell types (Carsience et al., 1993; Pain et al., 1996; Petitte et al., 1990). Their proliferation is dependent on the presence of several cytokines including LIF, stem cell, factor (SCF), insulin-like growth factor 1 (IGF1), interleukin 11 (IL-11) and basic fibroblast growth factor (bFGF) (Pain et al., 1996). Later work undertaken to simplify this medium showed that maintenance of proliferation can be achieved with avian LIF and serum (Horiuchi et al., 2004).

1.2.2.2. In vitro differentiation potential

Chicken ES cells have been reported to differentiate into cells of displaying markers of all three germ layers (Pain et al., 1996). Differentiation protocols for chick ES cells are based on those developed in their mouse and human counterparts. Removing components of medium that maintain proliferation of cES cells such as LIF slows proliferation and causes progressive loss of stem cell/pluripotency markers such as SSEA1, PouV and Nanog (Lavial et al., 2007;Pain et al., 1996).

Another method to direct cES cells to differentiate in vitro is to culture them on a low-adherence substrate in media depleted from growth factors that maintain proliferation (Doetschman et al., 1985). Under such conditions chick embryonic stem cells form three-dimensional structures similar to mammalian embryoid bodies (EB) (Pain et al., 1996). After being in the EB state in low-adherent culture dishes for a few days, cES cells are plated again on conventional tissue culture dishes where they spread and differentiate into cells of several lineages (Pain et al., 1996). Addition of chemicals such as retinoic acid can promote differentiation into a neural fate (Pain et al., 1996) which is accompanied by downregulation of markers such as Nanog and PouV/Oct3/4 (Lavial et al., 2007)

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1.2.2.3. Chimaera formation and germ line transmission

Injection of chick blastodermal cells into early embryos to make chimaeras was first done successfully many decades ago (Marzullo, 1970). This was repeated in different studies which confirmed that these blastodermal cells have the ability to contribute to somatic tissues as well as to the germ line (Carsience et al., 1993). Cultured cES cells can produce somatic chimeras with good efficiency (Pain et al., 1996) (See Table 1). However, the ability of blastodermal cells to contribute to the germ line is lost when these cells are cultured in vitro (Lavial and Pain, 2010; Pain et al., 1996; Petitte et al., 2004; Van de Lavoir et al., 2006a; Van de Lavoir et al., 2006b; Van de Lavoir and Mather-Love, 2006).

1.2.2.4. Molecular characteristics

Chick ES cells share many molecular characteristics with their mammalian counterparts (See Table 1) (Adewumi et al., 2007; The National Institute of Health, 2010). They include reactivity with antibodies raised against the mouse stage specific embryonic antigens (SSEA) 1, 3 and 4 (Knowles et al., 1978;Pain et al., 1996;Shevinsky et al., 1982;Solter and Knowles, 1978). These are used as markers for mammalian stem cell differentiation; for example, SSEA-1 is expressed in mouse embryonic stem (ES) cells and this expression decreases when cells are induced to differentiate (Kudo and Narimatsu, 1995). SSEA-3 and SSEA-4 are not present in proliferating mouse ES cells but are markers of the self-renewing state in human stem cells, where expression decreases with cell differentiation (Shevinsky et al., 1982). Other markers of chick ES cells that are shared with mammalian pluripotent stem cells include alkaline phosphatase activity (Lawson and Hage, 1994; Pain et al., 1996), and expression of mRNA for *PouV*(*Oct3/4*) and *Nanog* (Lavial et al., 2007) and *Sox2* (Bertocchini et al., 2010b) see below.

1.2.2.5. Transgenesis and prospects for genetic modification

The original aim of seeking methods for transgenesis of the domestic fowl was to create a bio-factory to produce biomedically valuable proteins in large quantities from egg albumen (Harvey and Ivarie, 2003; Ivarie, 2006; Sang, 2004). To date there has been only one successful attempt at gene replacement by homologous recombination in cES cells (Acloque et al., 2001). Other methods mainly rely on random integration of injected or transfected DNA or using a viral vector, to achieve transgenesis.

Some methods for generating transgenic birds have succeeded and several proteins have been generated this way including bacterial β-lactamase (Harvey et al., 2002) and human interferons (Rapp et al., 2003). Methods which have been used successfully to produce transgenic birds include microinjection of DNA into the early embryo (Love et al., 1994) and transfection by retroviruses or lentiviruses (Bosselman et al., 1989; Salter et al., 1987; Sang, 2004). Using the latter method, there are now lines of transgenic domestic fowl that ubiquitously express GFP (McGrew et al., 2004; Sang, 2004) and which have been used for example to follow cell lineage by transplantation, including a compelling demonstration that the tail bud of the early embryo contains endogenous stem cells that appear to be able to self-renew indefinitely, as serial transplants of the tail bud through 3 host embryos continue to generate axial and paraxial tissue (McGrew et al., 2008).

A few attempts have been made to generate transgenic chick ES cells using simple expression vectors (Pain et al., 1999) as well as gene trap vectors (Acloque et al., 2001). Other delivery systems can also be used to introduce transgenes into chick ES cells, such as cationic lipid-mediated DNA delivery which is now a standard transfection technique for cultured cells (Felgner et al., 1987; Felgner et al., 1989). However since it has not yet been possible to obtain contribution of ES cells to the germ line, these transgenic ES cell lines cannot yet be used to generate transgenic animals.

Table 1: Characteristics of ES cells in mouse, human and chick models

ES characteristics§	Mouse	Human	Chick	Chick Reference
'Stem' state markers				
SSEA-1	Yes	No	Yes	Pain et al., 1996
SSEA-3	No	Yes	Yes	Pain et al., 1996
SSEA-4	No	Yes	Yes	Pain et al., 1996
Sox2	Yes	Yes	Yes	Bertocchini et al., (In prep)
Oct3/4	Yes	Yes	Yes	Lavial et al., 2007
Nanog	Yes	Yes	Yes	Lavial et al., 2007
Enzymatic Activity				
Alkaline Phosphatase	Yes	Yes	Yes	Pain et al., 1996
In vitro culture requirement				
Feeder cells dependent	Yes	No	Yes	Pain et al., 1996
LIF dependent	Yes	No	Yes	Pain et al., 1996
Other characteristics				
Teratoma formation in vivo	Yes	Yes	Yes	Petitte & Yang 2004
Embryoid bodies formation	Yes	Yes	Yes	Pain et al., 1996
Tight rounded colonies	Yes	No	Yes	Pain et al., 1996
Loose flat aggregates	No	Yes	Yes	Pain et al., 1996

§Mouse and human data are based on the characterization of human embryonic stem cell lines by the International Stem Cell Initiative (Adewumi et al., 2007). A comprehensive and up to date list of ES cells markers can be found on the official National Institute of Health resource for stem cell research: Appendix E: Stem Cells Markers (The National Institute of Health, 2010).

1.3. Molecular regulation of self-renewal and pluripotency

The concept of totipotency/pluripotency slightly predates the first definition of stem cells. Its beginning stems from the classical embryological experiments done by (Driesch, 1891) on sea urchin. He showed that when a 2-cell-stage sea urchin embryo is cut in half it will develop into two complete larvae. This work was repeated in newt embryos of up to 16 blastomeres (Spemann, 1902). Totipotency is the ability of a cell to create any cell type or even an entire organism (Andrews et al., 2001). Pluripotency defines a cell that is able to give rise to many, but not all, embryonic tissues.

There is considerable interest in elucidating the mechanisms required to maintain pluripotency of ES cells, both for many potential biomedical applications related to tissue regeneration and to understand the mechanisms of commitment and differentiation and suppression of neoplasia. The following sections outline some factors and pathways that influence self-renewal and pluripotency in embryonic stem cells.

1.3.1. LIF pathway

An early finding was the discovery that activation of a cytokine pathway can alter both cell proliferation and potency. Sustained proliferation of mouse ES cells and the maintenance of the undifferentiated state in vitro was shown to be highly dependent on the leukaemia inhibitory factor (LIF; also known as differentiation inhibitory activity, Dia) (Niwa et al., 1998; Smith and Hooper, 1987). However it appears that LIF cannot prevent differentiation and support proliferation of human ES cells (Sato et al., 2004). Other factors are also required for sustaining pluripotentiality of ES cells in both species (Dani et al., 1998). Interestingly, in vivo studies confirmed that LIF is not required for pre-gastrulation mouse development (Nichols et al., 2001). This reminds us that embryonic stem cells are the product of in vitro culture, and that there is

probably no cell in the normal embryo at any stage that is entirely equivalent to an ES cell

1.3.2. Oct3/4 and its chick homologue POUV

Pluripotency of mouse ES cells seems to be tightly controlled by the POU transcription factor Oct3/4. The expression pattern of mouse Oct3/4 is limited to pluripotent cells (Pesce et al., 1998). During establishment of ES cell cultures from mouse embryos, this transcription factor is important for setting aside pluripotent founder cells (Nichols et al., 1998). If Oct3/4 expression is maintained at a critical level, it can prevent ESCs from differentiation upon withdrawal of LIF (Niwa et al., 2000). Several target genes have been identified for Oct3/4 in ESCs but little insight has emerged concerning their relevance to the regulation of multipotency (Niwa, 2001). POU family members act as transcriptional repressors or activators depending on a partnership with co-factors. Several co factors of Oct3/4 have been identified in the context of pluripotency regulation including adenovirus E1A which works as bridging factor between Oct3/4 and the basic transcription machinery (Scholer et al., 1991) and Sox2. The Oct3/4-Sox2 complex then auto controls the transcription of each of he two partners (Tomioka et al., 2002). In addition to this auoto-regulatory loop, the Oct3/4-Sox2 complex regulates several other target genes through differential enhancer activity (Botquin et al., 1998; Nishimoto et al., 1999; Nishimoto et al., 2005; Yuan et al., 1995).

In chick it was thought for a long time that there was no true homologue of Oct3/4 (Soodeen-Karamath and Gibbins, 2001). However more recently a gene named POUV was identified as the chick Oct3/4 homologue by sequence homology, synteny and functional conservation, since it can rescue mouse ES cells deprived of Oct3/4 (Lavial et al., 2007).

1.3.3. Nanog

Another key factor in the maintenance of self-renewal and pluripotency was identified relatively recently (Chambers et al., 2003). This gene encoding a

homeodomain-containing transcription factor was shown to maintain mESCs cell-renewal and pluripotency independently of LIF. The independent cloning of Nanog (Or Tir Na Nog, after the mythological Celtic land of the 'ever young') and subsequent functional analysis was a re-discovery of the function of this gene which has been originally described by another group which did not relate its function to stem cell self-renewal or pluripotency (Wang et al., 2003).

Although undifferentiated ESCs express Nanog, its normal levels do not prevent ES cell differentiation after withdrawal of feeders (Yasuda et al., 2006). Nanog seems to be one of several factors that are expressed in pluripotent cells and are downregulated during differentiation. Interestingly, Nanog was not among a group of 532 genes identified as being expressed in human ES cells (Brandenberger et al., 2004). Nanog expression was found to be responsible for the maintenance of the primitive ectoderm in the mouse embryo (Mitsui et al., 2003). In vitro, Nanog deficient mouse ES cells differentiate slowly into extra-embryonic endoderm lineages, which is consistent with the absence of a primitive ectoderm in $Nanog^{-/-}$ embryos (Mitsui et al., 2003). A similar induction of extraembryonic lineages is observed in ES cells following downregulation of Nanog in vitro (Darr et al., 2006b; Hyslop et al., 2005; Yates and Chambers, 2005). Over-expression of Nanog renders mouse ES cells resistant, but not completely refractory, to differentiation following LIF withdrawal or chemical induction (Darr et al., 2006a). The persistence of Nanog therefore seems to delay and increase the threshold rather than blocking ES cell differentiation.

Similar to Oct4, the level of Nanog per cell was found to be crucial for stable maintenance of an undifferentiated state; the reduced expression seen in $Nanog^{+/-}$ ES cells results in spontaneous differentiation after longer culture times (Hatano et al., 2005). Another similarity with Oct4 is the proposal that the Nanog acts by repressing the transcription of differentiation-promoting genes, suggested because Nanog-binding sites can be found in the control regions of genes like Rex1/Zfp42 (Mitsui et al., 2003).

An interesting finding on the regulation of Nanog concerns its relationship to the tumour suppressor p53, which binds to the Nanog promoter to maintain genetic stability of ES cells (Lin et al., 2005; Xu, 2005). Loss of p53 leads to a 100-fold increase in susceptibility to testicular teratoma which was proposed to be due partly to the repression of genes such as Nanog (Lin et al., 2005). And finally, Nanog was shown to interact with Smad1 to block BMP induced differentiation of ES cells: A gate keeper to the ES cells multipotent 'stem' state (Sun et al., 2006; Suzuki et al., 2006).

1.3.4. Sox2

Sox2 is a transcription factor (TF) belonging to the SoxB1 subfamily of genes (Miyagi et al., 2009). Sox proteins are characterised by the presence of an SRY-related high mobility group (HMG) box and act as transcription factors, probably able to cause physical bending of DNA. During early mouse development, Sox2 is first expressed in 2 sites and stages: first, it is expressed in the Inner Cell Mass (ICM) where its role was proposed to maintain cells in undifferentiated state (Wegner, 1999; Wood and Episkopou, 1999). Second, Sox2 is considered a general marker for the early neural plate (Graham et al., 2003; Rex et al., 1997a; Uwanogho et al., 1995; Wood and Episkopou, 1999; Zappone et al., 2000). Gene targeting experiments have revealed that Sox2 is required for very early embryonic development: Sox2 null mutant mice fail to develop beyond implantation (Wegner, 1999).

Sox2 is also expressed in at least three types of stem cells: neural, embryonic (ES cells) and trophoblast stem cells (Wiebe et al., 2000; Yuan et al., 1995). Sox2 is thought to be involved in maintaining pluripotency through its partnership with Oct3/4 (Chickarmane et al., 2006). This complex works as transcriptional activator for many genes implicated in maintaining pluripotency of ES cells such as FGF-4 and Fbxo15 (Tokuzawa et al., 2003) and Lefty1 (Nakatake et al., 2006). Through this mechanism the Sox2-Oct3/4 complex also regulates transcription of *Nanog* (Kuroda et al., 2005; Rodda et al., 2005). Furthermore, both *Sox2* and *Oct3/4* have regulatory regions with binding sites which are activated by the Sox2-Oct3/4 complex, which may define a positive

feedback loop involved in the maintenance of pluripotency in ES cells (Chew et al., 2005; Tomioka et al., 2002). Work on mouse ES cell pluripotency and differentiation demonstrated that factors that compete with Sox2 in binding to Oct3/4 are able to break this regulatory feedback loop leading to the exit of ES cells from the pluripotent self-renewing state and inducing differentiation (Niwa et al., 2005).

However, Sox2 appears to act redundantly with other factors in regulating transcription of pluripotency-maintaining factors: inducible $Sox2^{-/-}$ mutant ES cells have normal expression of such genes (Fgf4, Oct3/4, Nanog) (Masui et al., 2007). Nevertheless, these Sox2-null ES cells fail to maintain pluripotency and display alteration in the expression of factors that act upstream of Oct3/4 suggesting that the main role of Sox2 in ES cells is the maintenance of critical levels of Oct-3/4 expression. (Gu et al., 2005; Schoorlemmer et al., 1994). This could explain why Sox2 is not essential to maintain pluripotency when Oct3/4 levels are increased experimentally (Masui et al., 2007).

Studies in a broad range of eukaryotes have shown that transcriptional regulators that have key roles in cellular processes frequently regulate other regulators of the same processes (Guenther et al., 2005; Odom et al., 2004). It is likely that the key stem cell regulators bind and regulate genes encoding other transcriptional regulators, which in turn determine the developmental potential of these cells. We still lack sufficient knowledge of the regulatory circuitry of ES cells and of embryonic cells during development to understand this complex regulation fully.

1.3.5. iPS cells

A major recent discovery in stem cell research took place a few years ago when it was found that ES-like cells could be derived from tissues other than the inner cell mass of the early embryo. Mouse embryonic fibroblasts could be converted to cells with similar characteristics to ES cells by introducing just four transcription factors: Oct-3/4, Sox2, Klf4, and c-Myc. Cells so transformed were named Induced Pluripotent Stem (iPS) Cells (Takahashi and Yamanaka,

2006). Later it was discovered that iPS cells can be derived from human adult differentiated skin cells (Takahashi et al., 2007). The discovery that even adult somatic cells can be induced to acquire pluripotency and self-renewal comparable to those of embryonic stem cells immediately raised hopes that embryonic cells may become unnecessary for therapeutic applications, removing ethical objections raised by the general public and many religious groups as well as potentially offering a solution to immunological problems (potential rejection of heterologous cells)(Takahashi et al., 2007; Yamanaka, 2007).

1.4. Molecular mechanisms of neural specification

1.4.1. Neural Induction

Perhaps one of the most striking discoveries in developmental biology is the response of the ectoderm of the prospective belly of amphibian embryos to a graft of the dorsal lip of their gastrula blastopore – this results in the formation of an entire second axis containing a complete central nervous system derived from the host ectoderm. This experiment by Hilde Mangold and Hans Spemann (Spemann and Mangold, 1924) first demonstrated neural induction, as well as defining the “organizer” as an important embryonic signalling centre. The concept of embryonic induction i.e. an instructive interaction between two neighbouring tissues in which one changes its direction of differentiation in response to signals from the other, was proposed almost a century before Spemann and Mangold (von Baer, 1828) but lacked direct experimental demonstration. Moreover, Lewis (1907) had also previously shown that a secondary axis develops after grafting the dorsal lip of the blastopore. However, Lewis was unable to distinguish graft and host cells, a problem resolved by the use of interspecific grafts between differently pigmented newts

by Spemann and Mangold, which enabled them to show that the secondary axis originates from the host rather than from the graft.

Soon, other vertebrate Classes including birds (Waddington, 1932), teleosts (Luther, 1935; Oppenheimer, 1936) and mammals (Waddington, 1936; Waddington, 1937) were also shown to possess a region with similar activity to Spemann's organizer. Furthermore, organizers from a different Class can induce neural tissue. Chick Hensen's node (the functional equivalent of Spemann's organizer) grafts can induce a neural plate in fish(Hatta and Takahashi, 1996), amphibians (Kintner and Dodd, 1991) and mammals (Waddington, 1934). Likewise, rabbit nodes can induce neural tissue in birds (chick or duck) (Waddington, 1936; Waddington, 1937; Zhu et al., 1999). These interspecies transplantation experiments strongly suggest that the neural inducing signals are common to different vertebrates, suggesting conservation of the molecular mechanisms of neural induction.

The first molecular explanation for neural induction regulation came from the discovery that bone morphogenetic proteins (BMP) act as inhibitors of neural fate in Xenopus. Three genes, Noggin (Lamb et al., 1993), (Hemmati-Brivanlou et al., 1994) and Chordin (Sasai et al., 1994) expressed in the amphibian organizer were found to have a neuralizing activity through inhibition of BMP (Piccolo et al., 1996; Zimmerman et al., 1996).

These findings soon led to the 'default' model of neural induction. This model suggests that cells in amphibian ectoderm have an inherent 'default' tendency to become neural tissue, which is inhibited by BMP. BMP inhibitors secreted by the organizer lower the effective concentration of BMP in the dorsal ectoderm, allowing these cells to acquire a neural fate, whereas BMP concentration would remain high in more distant ventral regions, where the ectoderm becomes inhibited from assuming a neural fate and instead differentiates into epidermis (Hemmati-Brivanlou and Melton, 1997).

The wide distribution of BMP4 transcripts in the Xenopus blastula stage ectoderm (Fainsod et al., 1994), and the neuralization of Xenopus animal caps

when they are either injected with dominant-negative BMP receptors (Sasai et al., 1995; Xu et al., 1995), or treated with BMP antagonist Chordin and Noggin (Lamb et al., 1993; Sasai et al., 1995) as well as the dramatic loss of neural tissue and increase in skin formation when three of BMP antagonist (Chordin, Noggin, Follistatin) are depleted from Xenopus embryos (Khokha et al., 2005) all support the default model of neural induction.

Other work in Xenopus challenged the default model. BMP antagonist cannot neuralize ectoderm if Fibroblast growth factor (FGF) signalling is inhibited (Delaune et al., 2005b; Launay et al., 1996; Linker et al., 2009; Linker and Stern, 2004; Sasai et al., 1996). In the chick, neither BMPs nor their antagonists Chordin, Noggin or Follistatin are expressed in accordance with the predictions of the default model, misexpression of BMP cannot inhibit the expression of the early neural marker Sox3 in the neural plate and misexpression of BMP antagonists is not sufficient to induce neural tissue in competent ectoderm (Linker et al., 2009; Linker and Stern, 2004; Streit et al., 1998; Streit et al., 2000; Streit and Stern, 1999a; Streit and Stern, 1999b). These observations suggest that BMP antagonism is not sufficient for neural induction.

FGF signalling was implicated in neural induction by experiments in Xenopus showing that BMP antagonist fail to induce neural tissue under conditions blocking FGF signalling (Launay et al., 1996; Pera et al., 2003; Sasai et al., 1996), although findings from other groups made this controversial [eg (Amaya et al., 1991)]. Likewise in the chick several groups claimed that FGF was sufficient for neural induction (Alvarez et al., 1998; Henrique et al., 1997).

Eventually, through the cloning and characterisation of a very early marker for neural induction, ERNI, it was proposed that neural induction begins through FGF signalling even before gastrulation; moreover, inhibition of FGF signalling renders Hensen's node unable to neuralize competent epiblast (Streit et al., 2000). Time course experiments where an organizer was grafted, removed at various times and replaced by a graft of chordin (BMP-antagonist) secreting cells showed that BMP inhibition can stabilize the expression of early neural

markers induced by 5 hours' exposure to signals from the organizer, but cannot by itself induce neural tissue (Streit et al., 1998; Streit et al., 2000; Streit and Stern, 1999b). That FGF signalling is involved in initiating neural induction before gastrulation was also proposed by Wilson and Edlund (2000; 2001) based on the results of explant culture experiments.

It was then discovered that activation of the MAP kinase (MAPK) pahtway by FGF signalling can downregulate BMP targets via phosphorylation of the linker region of Smad1 reopened the controversy and questioning the independent role of FGFs in neural induction (De Robertis and Kuroda, 2004). However further work confirmed that FGF signalling is required for neural induction in both chick and Xenopus independently from its effects on BMP inhibition (Aubin et al., 2004; Delaune et al., 2005a; Linker and Stern, 2004).

Wnt signalling has also been implicated in neural induction in a paradoxical way. Studies in Xenopus suggested that Wnt activation is required for neural induction by inhibiting BMPs (Baker et al., 1999). However work using chick epiblast explants suggested that Wnt inhibition is required for FGF to induce neural tissue in epiblast cells as cells expressing or exposed to Wnt signalling take on epidermal fate (Wilson et al., 2001), a finding also supported by other experiments in Xenopus (Delaune et al., 2005a; Heeg-Truesdell and LaBonne, 2006). These paradoxical results could be explained by considering the timing of Wnt signalling during embryonic development. At very early stages, Wnt signalling is required specify the dorso-ventral axis of the embryo and help to position the organiser: high levels of Wnt specify dorsal fates. At a later stage Wnt signalling may have an antagonist effect on neural induction (Delaune et al., 2005a; Wilson et al., 2001).

Neural induction also involves other mechanisms and proteins. Churchill, a zinc finger transcriptional activator induced by FGF, was found to regulate cell choice between mesoderm and neural fate at the end of gastrulation (Sheng et al., 2003). Through its target genes (Sip 1: Smad-interacting protein 1 in chick and Xenopus and δ-EF1 in the mouse) (Miyoshi et al., 2006; Sheng et al., 2003; Snir et al., 2006) and interaction with POU domain genes (Nitta et al.,

2004) Churchill blocks expression of genes required for mesoderm formation such as Brachyury and Tbx6L, which stops the ingression of cells through the primitive streak at the end of gastrulation ensuring that the future neural plate remains on the surface.

Notch signalling (Yoon and Gaiano, 2006), POU transcription factors (Matsuo-Takasaki et al., 1999; Tanaka et al., 2004; Witta et al., 1995), as well as complex interactions between coiled-coil domain proteins (ERNI, Geminin and BERT), the heterochromatin proteins HP1α and HP1γ acting as repressors and the chromatin remodelling enzyme Brm acting as activator (Papanayotou et al., 2008) were all found to have roles in neural induction. Specification of embryonic epiblast cells to a neural plate fate is therefore the result of progressive decisions in response to a complex cascade of different signals (Stern, 2006).

1.4.2. Induction of neural differentiation in cultured ES cells

In vitro, ES cells can be induced to differentiate into neurons (Bain et al., 1995; Fraichard et al., 1995; Strubing et al., 1995). Several different protocols are available to direct ES cell cultures to a neural fate (Abranches et al., 2009; Studer, 2004). However we still have no idea whether commitment and differentiation to neural fates of these cells is an equivalent process to, or whether it is controlled by similar mechanisms than, neural induction in normal embryos.

For a relatively long period following the early reports, induced neural differentiation of ES cells was achieved by a two step method involving culturing the cells in suspension in low adhesion culture plates to form aggregates called embryoid bodies (EB) first, then plating them on conventional tissue culture dishes (Doetschman et al., 1985). This method directs ES cells through the complex interactions taking place within cells of the EB to differentiate into derivatives of all three embryonic germ layers (Doetschman et al., 1985).

To direct ES cells to a neural fate, EBs were grown in several conditions that seem to promote neural differentiation rather than differentiation into mesoendodermal lineages. EB were treated with various concentrations of retinoic acid (RA) (Bain et al., 1995) then plated on substrates that appear to promote neural or glial cell types such as gelatine (Strubing et al., 1995) or laminin (Bain et al., 1995). The result is rich networks of cells with neuronal morphology, expression of neural-specific proteins and electrical and chemical functionality (Bain et al., 1995; Liu et al., 2000). The mechanism through which RA can direct EB cells to a neural fate is not yet understood but may be related to its role in anterior-posterior patterning of the embryo through Hox genes (Krumalauf 1994) or to the recently discovered antagonism of FGF signalling by retinoids (Diez Del Corral et al., 2003).

Another successful enhancement of neuronal differentiation efficiency in EB based protocols was achieved by culturing cell aggregates in the hepatocarcinoma cell line (HepG2) (Rathjen et al., 2002). The result is absence of cells expressing mesoendodermal markers and a progeny expressing neural progenitor markers such as Nestin, Sox1 and Sox3. The mechanism by which HepG2 cells direct induced differentiation has not been identified (Rathjen et al., 2002).

Finally, a third strategy to enhance induced neural differentiation of EBs was described, based on growing EB in selective low serum-containing medium (Brustle et al., 1999; Lee et al., 2000; Okabe et al., 1996). Under these conditions most of the EB cells die and a small number of Nestin rich cells survives. The Nestin rich progeny could then be expanded and further directed to neuron-specific sub population by addition of factors with developmentally identified roles (See below).

An alternative to using EB cells relied on culturing ES cells on monolayers of bone marrow derived stromal cells (PA-6 line) (Kawazaki et al., 2000). This method of differentiation direct ES cells to differentiate into dopaminergic neurons by unidentified mechanisms (Barberi et al., 2003; Kawazaki et al.,

2002). However, as this neural effect is still seen when ES cells are cultured on fixed stromal cells, it has been proposed that induced neural differentiation is mediated through a cell surface mechanism rather than as a result of the production of cytokines by the feeder cells (Kawazaki et al., 2002).

Another method to induce neural differentiation is to culture ES cells on nonadherent (Tropepe et al., 2001) or adherent (Ying et al., 2003) substrates in the absence of feeder cells. A pre-requisite of this method is to grow ES cells in serum free in the absence of BMP signalling (Ying et al., 2003). Furthermore, ES cells derived from embryos in which components of the BMP pathway has been knocked out had almost a four-fold increase in induced neural differentiation suggesting that neural differentiation can be inhibited by BMP (Tropepe et al., 2001). This induced neural differentiation was shown to be dependent on FGF signalling. Blocking FGF signalling pharmacologically or using a dominant negative approach prevents neural differentiation of ES cells similar to what has been found in neural induction in the embryo (Streit et al., 2000).

Addition of factors to the basic medium has been used to study the effects of signalling pathways including Notch (Lowell et al., 2006), BMP (Tropepe et al., 2001) and FGF (Ying et al., 2003). Monolayer culture-based induced neural differentiation avoids the use of serum (Brewer et al., 1993; Ying et al., 2003; Ying and Smith, 2003) and the complex unidentifiable cell-cell interactions of other methods providing a useful system to define essential molecular mechanisms required to specify cell fate.

Altering the basic three methods of induced neural differentiation discussed above by adding signals known to control specific early patterning events in the embryo successfully led to directing ES cell differentiation into specific cell types. It was thus possible to direct ES cell induced neural differentiation into dopaminergic (Kawazaki et al., 2002; Lee et al., 2000; Ying et al., 2003), serotonergic (Kim et al., 2002), motor (Barberi et al., 2003; Mizuseki et al., 2003; Wichterle et al., 2002), GABAergic (Bain et al., 1995; Barberi et al., 2003) or glutamatergic neurons (Bain et al., 1995; Strubing et al., 1995) as

well as into several types of glial cells (Barberi et al., 2003; Brustle et al., 1999).

1.5. Sox2 and the SoxB1 family in neural development.

Sox (SRY-related HMG box) genes comprise a family of about 30 genes identified in relation to their role in sex determination in the male (SRY: sex-determining region of Y chromosome) (Kiefer, 2007; Wegner and Stolt, 2005). In mammals proteins encoded by these genes are organised into 8 groups, named A-H. This division is based on the phylogenetic characteristics of a highly conserved 79-amino acid high mobility group (HMG) box domain with sequence-specific DNA binding function (Bergstrom et al., 2000; Dailey and Basilico, 2001; Soullier et al., 1999).These proteins bind to DNA in a sequence specific manner and act as either activators or repressors of transcription of other proteins (Ambrosetti et al., 1997; Ambrosetti et al., 2000; Catena et al., 2004; Kamachi et al., 2001; Krstic et al., 2007; Mojsin and Stevanovic, 2010; Tsuruzoe et al., 2006).

The second group (B) is divided into two subgroups: SoxB1, which comprises the transcription factors Sox1, Sox2, and Sox3 and SoxB2 which includes Sox14 and Sox 21 (Holmberg et al., 2008; Miyagi et al., 2009). The SoxB1 subfamily of transcription factors has been implicated in many developmental processes as regulator of cell fate decisions [reviewed by (Miyagi et al., 2009)]. Here, their role in neural development is discussed with a focus on Sox2.

In addition to its expression in the early epiblast of vertebrate embryos (Graham et al., 2003; Wood and Episkopou, 1999; Zappone et al., 2000) Sox2 is also expressed in the early neural plate (Collignon et al., 1996; Graham et al., 2003; Rex et al., 1997a; Uwanogho et al., 1995; Wood and Episkopou, 1999; Zappone et al., 2000). Sox2 is considered one of the earliest definitive markers for the neural plate (Albazerchi and Stern, 2007; Linker and Stern,

2004; Papanayotou et al., 2008). In addition to the timing and extension of expression domain itself, time-course experiments have revealed that Sox2 is induced after exposure to organizer-derived signals after a period of time similar to that required to induce a mature neural plate (Bainter et al., 2001; De Robertis and Kuroda, 2004; Stern, 2005a; Streit et al., 1998; Streit and Stern, 1999a) .

Expression of the other SoxB1 group members (Sox1 and Sox3) overlap extensively with Sox2 in the developing CNS in both rodents and birds (Graham et al., 2003; Pevny et al., 1998; Uwanogho et al., 1995; Wood and Episkopou, 1999). It is therefore difficult to get insight into a differential role of the SoxB1 family members based on their expression patterns.

All members of the SoxB1 family have been targeted by loss of function ('knock out') experiments. Sox1 homozygous mutants live and display no major CNS abnormalities apart from lens defects (Malas et al., 2003). In contrast, loss of Sox2 or Sox3 lead to embryo lethality (Rissoti et al., 2004; Wegner, 1999).

Induced loss of Sox2 function in mouse embryos shortly after gastrulation reveals its essential role in the development of the anterior CNS and the retina as embryos lacking its expression at this stage develop with enlarged lateral ventricles of the cerebrum (Graham et al., 2003) and complete loss of proliferation and differentiation of neural retinal progenitor cells (Taranova et al., 2006). The defects caused by the loss of Sox2 function can be rescued by expression of Sox. Furthermore, the level of Sox3 expression was elevated in the Sox2 mutant embryos, suggesting possible partial functional compensation by other SoxB1 genes upon loss of Sox2 (Bylund et al., 2003; Ferri et al., 2004)

Conditional Sox3 loss of function leads to a spectrum of phenotypes (about one third of embryos appear normal). Defects observed in abnormal embryos include problems in the development of the hypothalamo-pituitary axis (Rissoti et al., 2004).

The defects observed in different aspects of CNS development, especially in the developing eye where the overlap between Sox1, 2 and 3 is less than that in rest of the CNS, gives a unique insight of the differential and redundant functions between SoxB1 proteins. Sox3 is never expressed in the lens, and Sox2 expression is restricted to early stages of lens development. This functional redundancy between members of the SoxB1 family appear to be a conserved biological process from fly to mammals (Ferri et al., 2004; Graham et al., 2003; Overto et al., 2002; Wegner, 1999).

In cultured cells, Sox2 is important for the differentiation of embryonic neural stem cells into mature neurons whereby neural progenitors derived from Sox2 knocked down embryos fail to differentiate into mature MAP-2 positive cells in a way that could be rescued by over expression of Sox2 (Cavallaro et al., 2008).

Two fundamental characteristics of SoxB1 group regulatory mechanisms have already been revealed. First: Sox proteins bind to other transcription factors to form complexes to regulate many target genes during development (Kamachi et al., 2000). Examples of this include the Sox2-Oct3/4 complex which maintains the stem state of ES cells (see above) and the interactions between Sox2 and Pax6 in eye development (Inoue et al., 2007).

Second, the expression of these transcriptional factors (as shown for Sox2) is regulated by multiple enhancers which are activated by specific factors in different cell types and distinct stages of embryo development (Uchikawa et al., 2003). These enhancers are highly conserved between vertebrate species (Uchikawa et al., 2003; Wegner, 1999).

Five enhancers (named N1-N5) are responsible for directing Sox2 expression to different parts of the CNS at different times in development (Kamachi et al., 2009; Kondoh and Uchikawa, 2008; Uchikawa et al., 2003). In the chick embryo, the N2 enhancer controls Sox2 expression in the early anterior neural plate when Sox2 expression marks commitment to a neural plate fate

(Uchikawa et al., 2003). The same N2 enhancer was found to activate Sox2 transcription in proliferating ES cells as well as mouse neural progenitor cells in the mouse embryo (Miyagi et al., 2004; Tomioka et al., 2002; Zappone et al., 2000). In mouse ES cells it was demonstrated using gel shift assays that Oct3/4 binds to this site to activate Sox2 transcription (Catena et al., 2004; Tomioka et al., 2002; Zappone et al., 2000), whereas the related POU factors Brn1/2/4 and Oct-6 activate Sox2 mRNA expression in the mouse embryo nervous system (Catena et al., 2004).

Analysis of the N2 enhancer identified multiple putative binding sites for known transcription factors (Uchikawa et al., 2003; Uchikawa et al., 2004)(also see appendix 1). Recent functional experiments in the chick embryo revealed that Otx2 (Kamachi et al., 2009) and a group of coiled-coil proteins interact with each other and with chromatin-remodelling factors and heterochromatin proteins to regulate the activity of the N2 enhancer to regulate the expression of Sox2 in precursors of the anterior neural plate (Kamachi et al., 2009; Papanayotou et al., 2008). Studies such as these allow dissection of the critical factors, and thereby the molecular networks responsible, for cell fate decisions during both normal embryonic development and in cultured pluripotent embryonic stem cells.

1.6. Aims of this thesis

Stem cells are emerging as an important tool to study biological processes, investigate the aetiology of disease and design pharmaceutical and cell based therapies (Smith, 2001). Among the diseases for which there is hope for the latter are neurological conditions, especially neurodegenerative disorders such as Parkinson's disease where cell replacement could offer substantial hope (Chiba et al., 2008;Wernig et al., 2008).

To harness the power of multipotent embryonic cells to generate neural tissue for transplantation, it is essential to understand the mechanisms directing their differentiation both during normal embryonic development and in cultures of

embryonic or adult-derived stem cells. In the embryo, we are only just starting to understand neural induction, which appears to be a complex multistep process. It is now believed to start before gastrulation and ends in the formation of the embryonic neural plate (Stern, 2004b; Stern, 2005a; Stern, 2006). At the latter stage, expression of transcription factors of the SoxB1 Class, and especially Sox2 and Sox1, represent good markers for this state as soon as the neural plate can be identified in the embryo (Collignon et al., 1996; Ellis et al., 2004; Linker and Stern, 2004; Papanayotou et al., 2008; Rex et al., 1994; Rex et al., 1997b; Uwanogho et al., 1995). More recently it was discovered that Sox2 also plays a key role in regulating the self-renewal of ES cells (Avilion et al., 2003).

Sox2 is expressed in many sites during development and at different stages (Wegner, 1999). Recent studies on how this complex expression is regulated have revealed that different aspects of its expression are controlled by 25 highly conserved non-coding elements (Uchikawa et al., 2003). Between them, 5 of these enhancers (N1-N5) account for the expression of Sox2 in the developing CNS. The remaining non-coding elements direct expression to other tissues especially components of the PNS, such as neural crest and placodes (Uchikawa et al., 2003). However we still know very little about the mechanisms that direct Sox2 expression in proliferating ES cells.

It is difficult to study neural induction in the mouse, which develops in utero and cannot be cultured in vitro at peri-implantation stages. For this the chick has been a more useful model and much progress has been made recently as reviewed in (Stern, 2004b; Stern, 2005a; Stern, 2006). However ES cells had only been well characterised in the mouse. The relatively recent isolation of chicken embryonic stem cells (cES cells) (Pain et al., 1996) now allows the use of the same animal model to study neural induction and the regulation of Sox2 expression in vivo and in vitro. This project represents an initial characterisation of cES cells by studying the dynamics of their differentiation and the mechanisms responsible for Sox2 expression in the self-renewing (“stem”) state and during induced neural differentiation.

As this project is based on in vivo studies in the embryo (Catena et al., 2004; Uchikawa et al., 2003; Zappone et al., 2000), studying the regulation of Sox2 in cES cells raises two fundamental questions: Is the process of induced neural differentiation of cES cells in any way comparable to neural induction in the embryo? Are these processes controlled by similar mechanisms?

The aim of this project will, therefore, be to answer the following questions:

1] What is/are the enhancer(s) which regulate Sox2 mRNA expression in cES cells during their prolifrative 'self-renewal' condition?
2] Can cES cells be differentiated into neurons in a way comparable to methods described in their mammalian counterparts?
3] Given the hypothesis that cES cells can be induced into neuronal differentiation in vitro in a way similar to mammalian ES cells, would Sox2 expression change during this induced neural differentiaion?
4] What is/are the enhancer(s) which regulate Sox2 mRNA expression following induction of differentiation to a neural fate?

To answer these questions, Chapter 1 has reviewed the literature by introducing ES cells in general with a focus on cES cells, regulation of the 'stem' state, the 'neural' state as well as the neurodevelopmental role played by Sox2 and the rest of the members of SoxB1 family of transcription factors.
.
After describing the principal methods used in this project (Chapter 2), Chapter 3 examines the activity of conserved non-coding genomic regions associated with Sox2 in proliferating cES cells and reveals the N2 enhancer as the most critical element. Chapter 4 compares different protocols for inducing cES cells to acquire a neural fate. Chapter 5 then follows the changes in Sox2 expression as well as the activity of the N2 enhancer over a 10 day period following initial induction of neural differentiation. A further refinement of the region driving expression of Sox2 reveals a small core, both necessary and sufficient for the activity of the N2 enhancer.

Chapter 2. Methods

2.1. Preparation of culture dishes and feeder cells

The method used in extracting and maintaining cES cells is based on a a modified version of a patented method registered in the United States Patent and Trademark Office No. 5,340,740 (Petitte and Yang, 1994). Dishes (Becton Dickinson, USA) were coated with 0.1% gelatine in sterile water solution (Chemicon, USA). 2 ml of the gelatine solution were added to each well of a 6 well dish, 1 ml/well for 12 well dish, 0.5 ml/well for 24 well dishes, and 0.3 ml/well for 48 well dishes. The wells were incubated at room temperature for 30 minutes, and the gelatine removed before use.

STO feeder cells (American Type Culture Collection No. CRL 1503) were prepared by maintaining the cells at 37°C in 7.5% CO2 in DMEM containing 10% FBS and 2 mM L-glutamine. The cells were treated with 10 µg/ml of Mitomycin C (Sigma) for 90 minutes at 37 °C, rinsed with PBS then detached from the culture dishes using 0.25% trypsin/0.025% EDTA solution (GIBCO™). The trypsin was inactivated by collecting the cells in DMEM with 10% fetal bovine serum (FBS) and washed at 1100 rpm for 10 minutes. After washing, cells were resuspended in DMEM with 10% FBS and counted. The cells were then seeded on gelatinized plates as described above at a density of $1x10^5/cm^2$ and incubated for one to four days before use.

2.2. Preparation of conditioned medium

Stem cells were maintained in Buffalo Rat Liver (BRL) cell conditioned medium supplemented (See below) to make Embryonic Stem cell culture medium (ESA). First, BRL-3A cells (American Type Culture Collection No.

CRL 1442) are cultured and expanded in DMEM (GIBCO™, UK) containing 10% FBS and 2 mM L-glutamine. After BRL cells were grown to confluence, the primary medium is replaced with knockout DMEM containing 5% FBS and 2mM L-glutamine and cells cultured at 37°C in 5% CO2 for 3 days. The secondary (conditioned) medium was removed and two new batches of medium were conditioned for another 2 periods of 3 days each. The conditioned medium is then filtered and diluted to 50% or 80% with knockout DMEM with 15% FBS and this then supplemented with: 1 mM sodium pyruvate (GIBCO™ , UK), 1% MEM Non-Essential Amino Acids Solution 10 mM (100X) (GIBCO™ ,UK), 1% MEM Vitamin Solution (100X) (GIBCO™ , UK), 1 mM of each nucleotide (adenosine, guanidine, cytosine, uridine, thymidine; Chemicon, USA), 0.16 mM b-mercaptoethanol (Chemicon, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO™ , UK) to make up the embryonic stem cell culture medium (ESA). Media were stored at 4°C and used within 7 days.

2.3. Generation of cES Cells from avian blastodermal cells

Freshly laid unincubated eggs (Brown Bovan Gold; Stewart UK) were used. Embryos were staged according to (Eyal-Giladi and Kochav, 1976). Only embryos prior to the formation of the primitive streak - preferably at stage X-XI - were used to prepare the blastodermal cells. The entire blastoderm was removed and dissociated by gentle aspiration with a Pasteur pipette in phosphate buffered saline solution (PBS) at room temperature. Embryo dissociated blastodermal cells were then pooled at 1 embryo per ml and centrifuged at 400 g for 10 minutes. The cell pellet was then gently suspended in ESA complete medium. Cells were seeded at a final concentration of 1 embryo per well in ESA complete medium on gelatine coated 48 well dishes which had been seeded with inactivated STO feeder cells. The blastodermal cells were maintained at 39°C in 7.5% CO_2. One-quarter of the medium was replaced with fresh medium after 24 hours in culture. Half of the medium was then changed on the third day and all of it every day thereafter.

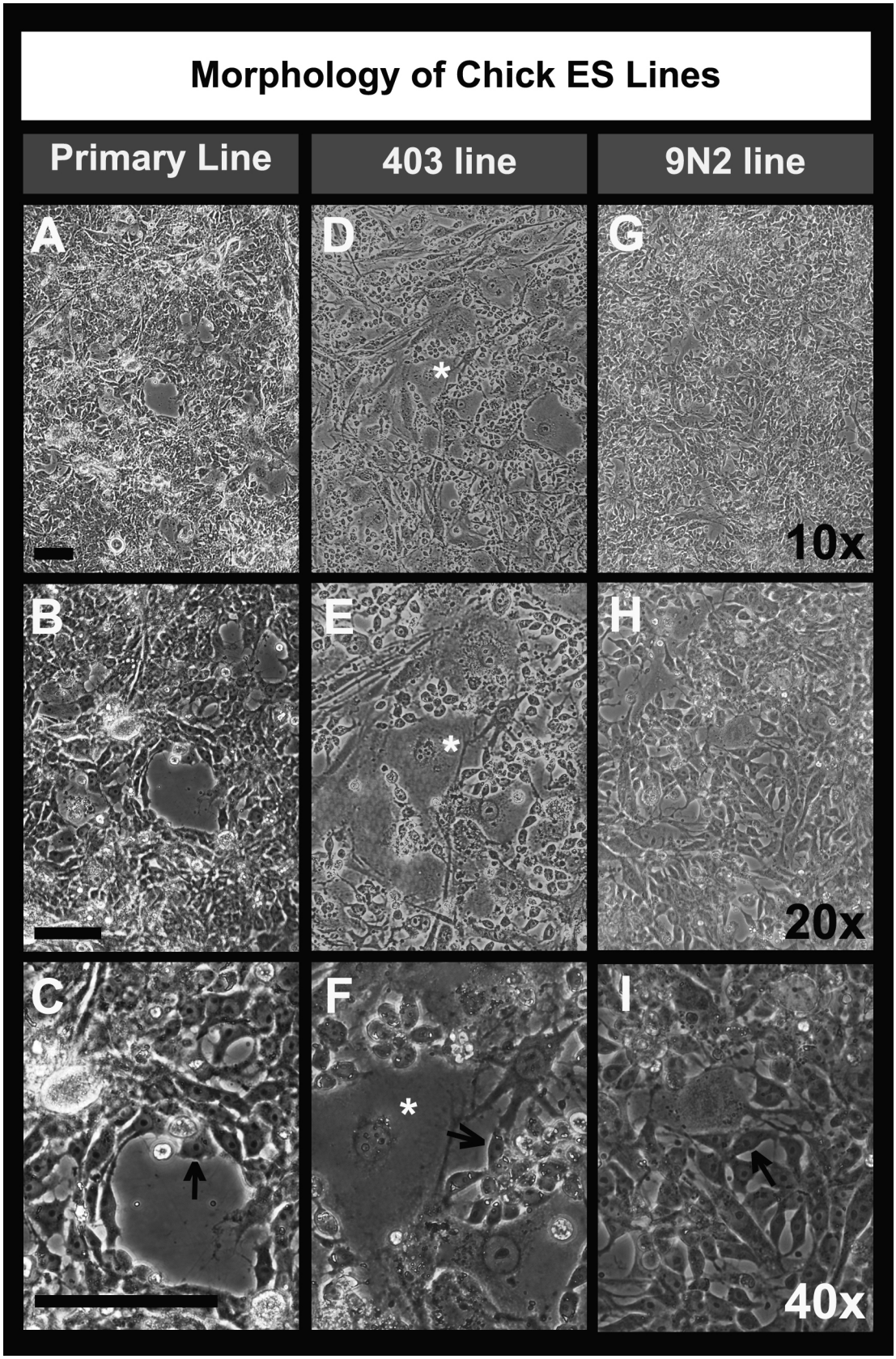
2.4. Maintenance of cES cells and cell lines

Cryo-preserved cES Cells from established lines (see below) were thawed and maintained in culture according to published protocols (Pain et al., 1996, van de Lavoir and Mather-Love, 2006). The cells were grown on a STO feeder layer with a concentration of cES: STO of 10:1. The cES cells were cultured until 80–100% confluence before they were passaged in a 1:2 – 1:3 ratio. Usually, cultures were passaged daily. To maintain optimum growth and to prevent differentiation, the cells were passaged by transferring 25–30% of the medium covering the cells into the new well. The cells were then dispersed using either a short trypsin (0.25% trypsin/ EDTA solution diluted to 20% in PBS solution) wash or 1–2 min incubation in Ca/Mg free PBS and passaged as small clumps. The morphology of the cES cells was observed daily to confirm that they remained undifferentiated, using the criterion of a large nucleus with a prominent nucleolus and relatively little cytoplasm (Figure 2.1).

Two established chicken embryonic stem cell lines were used: 9N2 (Acloque et al., 2001), 403 (Origen Therapeutics, Inc.1450 Rollins Road, Burlingame, California 94010, USA) and a primary line (30) which was generated and characterized in Professor Claudio Stern lab Ms. Sharon Boast in 2005. All three lines resemble murine and human embryonic stem cells (see Table 1) and can all be maintained as described above.

Figure 2.1: Morphology of cES cells: Three cES cell lines morphology is displayed at 10x, 20x and 20x objective lens magnifications. Following extraction of blastodermal cells from the epiblast of freshly laid eggs and culturing them for several days in ESA medium, the morphology of the cES cells is observed daily to confirm yield of primary cell line. A-C: Successful yield of a primary cES cells with typical characteristics of clumps of tear-drop-like cells with prominent large nucleus and relatively little cytoplasm (arrows). These can easily be distinguished from large and flat feeder cells (asterisk) displaying characteristic clumps/clusters of cells with little cytoplasm, large nucleus and prominent nucleolus (arrow heads). These primary cES cells resemble those of established lines such as the 403 (D-F) (Origen Therapeutics, Inc.1450 Rollins Road, Burlingame, California 94010, USA) and the 9N2 (G-I) (Acloque et al., 2001) lines. (Scale bar = 80μm).

Figure 2.1: Morphology of cES cells



2.5. Reporter constructs for Sox2 enhancers

Kindly gifted from Professor Hisato Kondoh of the Graduate School of Frontier Biosciences, Osaka University Japan, the Plasmids which were used to identify active enhancer regions of *Sox2* were based on the plasmid tk-EGFP reporter vectors used in the original study (Uchikawa et al., 2003). These plasmids were constructed by insertion of the Herpes simplex virus thymidine kinase promoter in the polylinker HindIII site of pCAT3-basic vector (Promega), and replacing the CAT gene with the EGFP gene (Clontech). Twenty-six plasmids each contains one conserved regulatory element upstream and downstream of the chick Sox2 locus (Uchikawa et al., 2003) were tested (See Figure 2.2, Table 2).

The regulatory sequence was inserted in the polylinker SmaI site of ptkEGFP. The chromosomal locations and other characteristics of these sequences are shown in (Table 2). As a control for electroporation, pCAB, a plasmid containing the chicken β-actin promoter driving GFP, was used. A 'negative control' promoter was also used to identify the baseline activity of the minimal promoter following transfection. This plasmid contained the minimal promoter thymidine kinase (tk) and the EGFP gene with no enhancer sequence inserted upstream of the tk promoter.

Figure 2.2: Plasmids used for testing the activity of Sox2 enhancers in cES cells: Twenty-six plasmids each contains one conserved regulatory element located upstream and downstream of the chick Sox2 locus (Table 2) (Uchikawa et al., 2003) were tested. These plasmids were constructed by insertion the conserved regulatory element upstream of the Herpes simplex virus thymidine kinase promoter. This complex would identify the enhancers of these regulatory sequences by driving the expression of the reporter gene EGFP (Clontech) if the regulatory sequence had an activity in transfected cells.

Figure 2.2: Plasmids used for testing the activity of Sox2 enhancers in cES cells

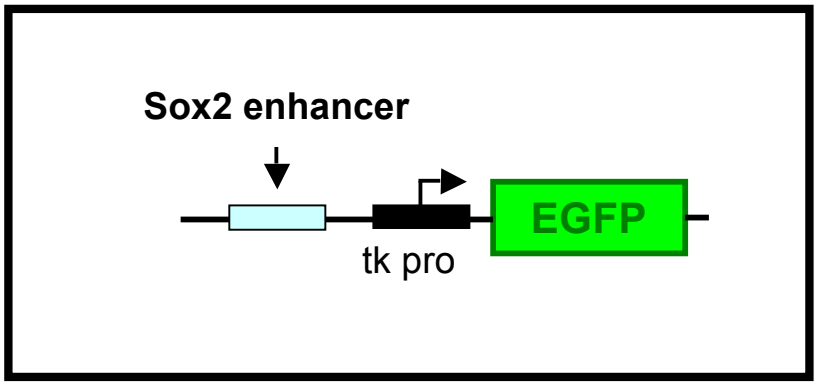


Table 2: Conserved sequences of Sox2 across amniote species

Block	Bp*	Assigned Enhancer	Specificity of Enhancer	Earliest Stage¶ of Activity	Length* in Chicken genome	Position in Chicken genome¥	Homology in Human (%)	Homology in Mouse (%)
1	438				496	-16175	77	68
2	639	N3	DC/MC/Early lens	8	585	-15133 t	89	71
3	509				487	-11102	62	Absent
4	891				875	-10068	71	68
5	334	NOP-1	Nasal/Otic	11	323	-8811	83	81
6	308				145	-7803	75	68
7	179				143	-5310	75	71
8	550	N2	TC, MC, DC	5*	534	-3012	90	87
9	147				147	-1198	84	81
11	103				103	3427	63	61
12	295				287	4314	83	83
13	395	NOP-2	Nasal/Otic	11	374	5105	85	77
13.5	201				138		Absent	Absent
14	395	N-5	RC; r-2, r-4	12	366	8151	75	72
15	205				188	9872	71	71
16	358				344	10445	87	86
17	324	N-1	Anterior PS	5*	298	12825	83	72
18	415	SC-1	SC		413	13666	81	65
19	394				362	15041	84	80
20	208				127	16389	74	61
21	312				292	16988	61	Absent
22	169	SC-2			171	18450	70	69
22.5	201				161		62	Absent
23	171				158	20551	67	(55)
24	556	N4	Head	10	480	22339	78	73
25	700	NC-1	Dorsal root ganglia	17	676	29610	85	Absent

***** Sequence block length in the Plasmid (base pairs)

¶ Stages are according to (Hamburger and Hamilton, 1951) table.

¥Position is in relation to *Sox2* genomic locus. Negative sites refer to sequences positioned upstream of Sox2 locus.

PS: primitive streak, SC: Spinal cord,

RC: Rhombencephalon, TC: Telencephalon, MC: Mesencepalon, DC: Diencephalon

2.6. Transfection with LIPOFECTAMINE™2000

Based on methods that were developed by (Felgner et al., 1987; Felgner et al., 1989), Lipotransfection with Lipofectamine™2000 was used to transfect DNA into cES cells in 24-well plates. For other well sizes, a scaling table (Table 3) was used. Transfection method was optimized in relation to transfection efficiency and cell survival using different Lipid:DNA ratios. Figure 2.3 and Figure 2.4 show the outcome of experiments used to optimize the lipotransfection procedure. Following optimization, all transfection experiments reported in this thesis used a Lipofectamine ™ 2000:DNA ratio of 3:1 (µg/µl).

The transfection process would start by seeding the feeder layer cells at a density of 10^4 cells/cm^2 and incubated at 37ºC overnight. The following day, cES cells were plated at a density of 10^5 cells/cm^2 and incubated in BRL conditioned medium for 18-24 hours. For each transfection, two 1.5 ml tubes were prepared: in the first 1 µg DNA was diluted in 99 µl of Opti-MEM® I reduced serum. In the second 3 µl LIPOFECTAMINE™2000 was diluted with 97 µl of Opti-MEM® I reduced serum and incubated at room temperature for 5 minutes. The solutions of the two tubes are then combined and incubated at room temperature for 20 minutes. The combined solution (total of 200 µl) is then added to each well, mixed gently and incubated for 6 hours at 37ºC. After 6 hours, one ml of fresh ESA medium was added and incubation continued overnight. On the first day following transfection (18-24 hours following transfection) the transfected cES cells were examined under fluorescence illumination to check for GFP expression. Cells' culture medium was changed and cells incubated for another day. On the second day post transfection (36-48 hours following transfection), the medium was changed and cells were checked again for GFP expression, counted, recorded in the spreadsheet, and the photographs were taken before cells were fixed in 40% paraformaldehyde (PFA) in phosphate buffered solution (BPS).

Table 3: Scaling of transfection volumes with Lipofectamine TM 2000 for different sizes of wells

Culture Dish	Surface area (cm^2) (per well)	Shared Reagents		DNA transfection	
		Volume of plating medium	Volume of dilution medium	DNA (µg)	Lipofectamine™ 2000 (µg)
96 well	0.3 cm^2	100 µl	2 x 25 µl	0.25 µg	0.75 µl
24 well	2 cm^2	500 µl	2 x 50 µl	1 µg	3 µl
12 well	4 cm^2	1 ml	2 x 100 µl	2 µg	6 µl
6 well	10 cm^2	2 ml	2 x 250 µl	4 µg	12 µl
60 mm	20 cm^2	5 ml	2 x 0.5 ml	8 µg	24 µl
10cm	60 cm^2	15 ml	2 x 1.5 ml	24 µg	72 µl

Figure 2.3: Optimizing lipotransfection of cES Cells: The sequential Lipofectamine^{TM}2000:DNA ratios 3:1, 4:1, 5:1 were tried. Transfection efficiency, assessed by the number of green fluorescent cells 36 hours following transfection, was as high with Lipid:DNA ratio 3:1 (A,D,G and Figure 2.4) was as high as that observed following transfection with 4:1 (B,E,H) and 5:1 (C,F,I) ratios. Cells' survival, assessed by number of cells in CM^2 on the following day following EGFP assay (60-72 hours following transfection) as an indicator of cells' ability to continue proliferation following transfection, was related to the amount of Lipofectamine^{TM}2000 used. Decreased cell counts (likely due to Lipofectamine^{TM}2000 toxicity) were observed when higher Lipid:DNA ratios were used (compare J with K,L) (Scale bar = 80μm).

Figure 2.3: Optimizing lipotransfection of cES Cells

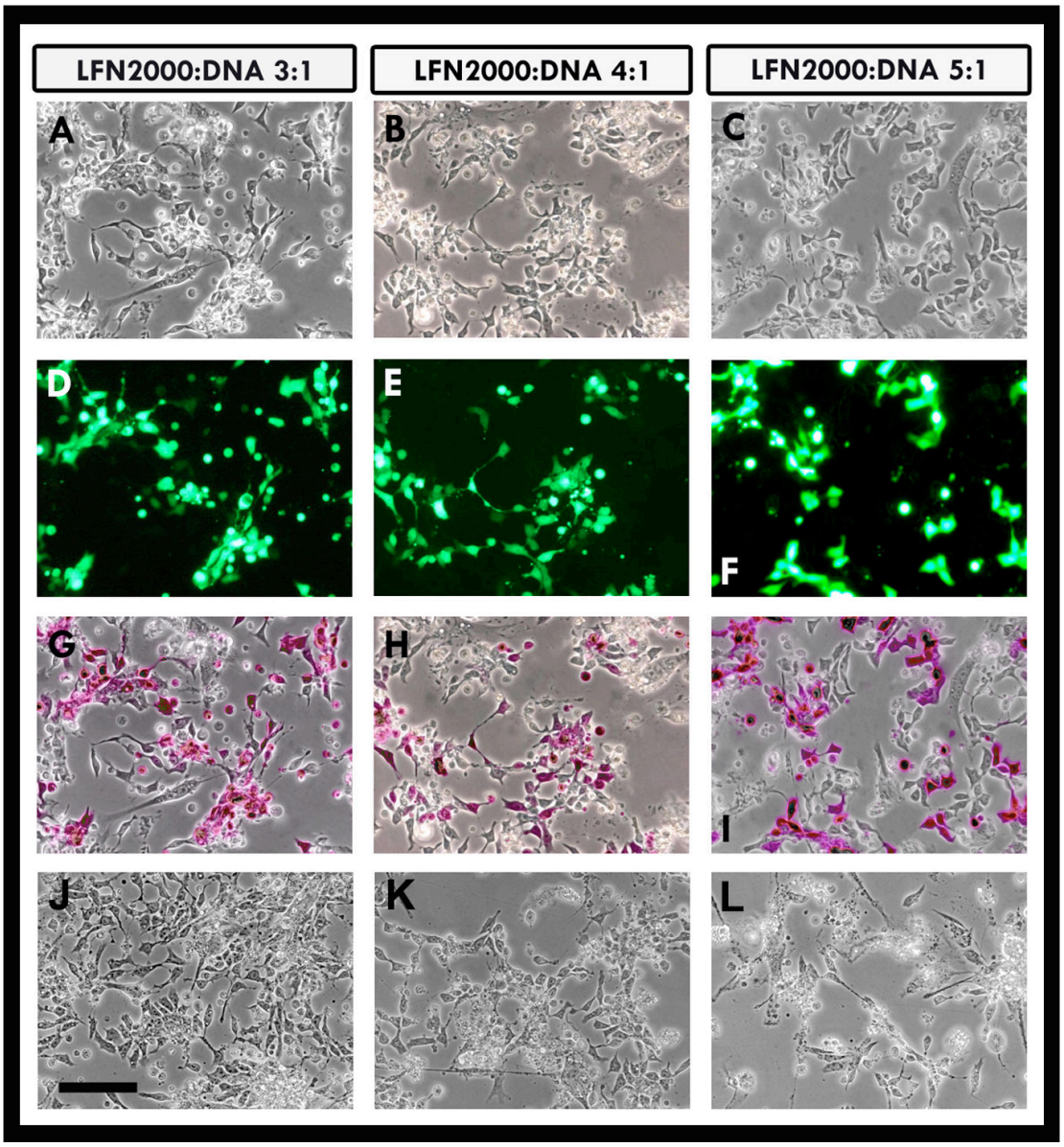
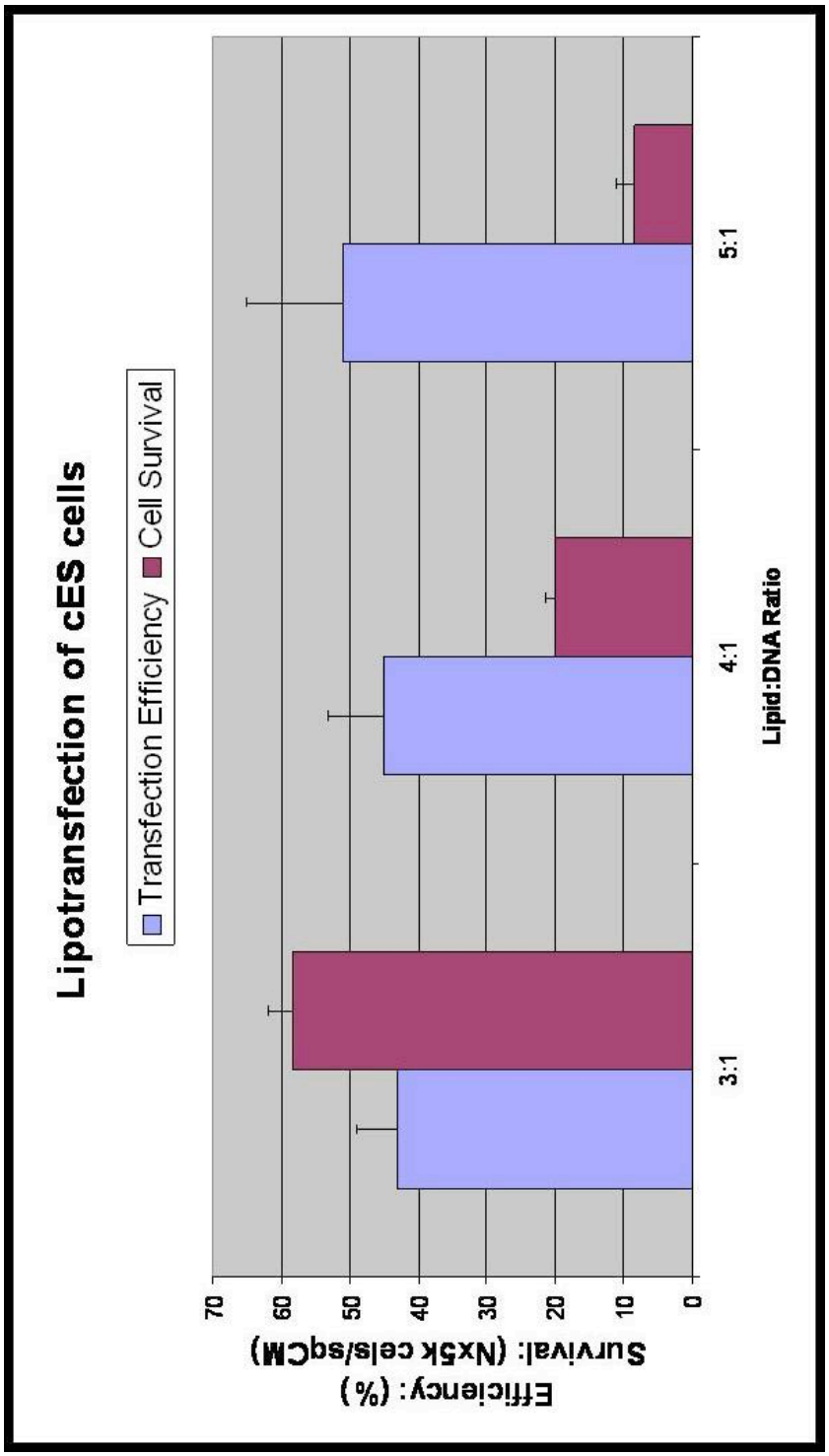


Figure 2.4: Lipotransfection of cES Cells - Efficiency vs Survival: The sequential Lipofectamine^{TM}2000:DNA ratios 3:1, 4:1, 5:1 were tried. Transfection efficiency, assessed by the number of green fluorescent cells 36 hours following transfection, was as high with Lipid:DNA ratio 3:1 as that observed following transfection with 4:1 and 5:1 ratios. Cells' survival, assessed by number of cells in CM^2 on the following day following EGFP assay (60-72 hours following transfection) as an indicator of cells' ability to continue proliferation following transfection, was related to the amount of Lipofectamine^{TM}2000 used. Decreased cell counts (likely due to Lipofectamine^{TM}2000 toxicity) were observed when higher Lipid:DNA ratios were used (See also Figure 2.3 above) .

Figure 2.4: Lipotransfection of cES Cells - Efficiency vs Survival



2.7. Cell counting and basic statistical analysis methods

To determine the activity of the reporter constructs, cell survival and mRNA expression, related cells (eg green fluorescent cells following transfection) were counted using a 20x objective in an Olympus Vanox-T microscope with epi-fluorescence optics for GFP. The total number of cells in the same field was counted using phase contrast optics. Tissue culture wells of each experiment were blindly selected. For each well, six separate fields spanning the horizontal equator of the well were recorded, transferred to a spreadsheet before performing statistical analysis to report the characteristics of these relevant cells (eg mean ± standard deviation of green fluorescent cells)

Analysis of variance (ANOVA) was used to assess statistical significance in the number of GFP-expressing cells between different plasmids with Bonferroni adjusted post-hoc tests' analysis to make individual comparisons between two individual set of scores. A P value of 0.05 was accepted as statistically significant. All statistical analyses were performed using SPSS for Windows, Release 14 (SPSS Inc.).

2.8. In situ hybridization (ISH) of chick embryonic stem cells (cESCs).

2.8.1. Transcription of DIG-riboprobe

Vectors were cut with the appropriate restriction enzyme for 4-5 hours or overnight and verified by agarose gel electrophoresis using 1/20th of the initial reaction volume. The DNA was then extracted with Phenol:Chloroform followed by Na-Acetate/Ethanol precipitation after which the DNA was dissolved at about 1mg/ml. The DIG-riboprobe was then transcribed with the appropriate enzyme (T3, T7 or SP6) at 37 ^{o}C for 2 hours (for SP6 transcription 2-3 times the amount of DNA template was used and transcription done at 40^{o}C). The remaining DNA template was then digested with DNase I for 30 minutes and the DIG-riboprobes checked by agarose gel electrophoresis. The probe was precipitated with Lithium Chloride and ethanol, washed in 70% ethanol and re-dissolved in water at about 1 mg/ml. This was then further diluted about 5-10x (to 100-200 µg/ml) in hybridization buffer for storage at -20 ^{o}C.

2.8.2. Preparation of cESCs for ISH

Cells were fixed in freshly made 4% paraformaldehyde (PFA) in BPS for 15 minutes at 4 ^{o}C. PFA is then replaced with absolute methanol and cells stored in this for up to 1 week at -20 ^{o}C. If cells needed to be kept longer before in situ, they were taken through the first day ISH procedure (below) until just before adding the probe and then kept at -20 ^{o}C until needed.

On the first day, cells were rehydrated through 75%, 50% and 25% methanol in Calcium Magnisium Free (CMF) PBS containing 0.1% Tween-20 (PTW) at room temperature and washed twice with PTW at room temperature for 5 min.

Cells were then post-fixed for 30 minutes at room temperature in 4% paraformaldehyde in PTW containing 0.1% glutaraldehyde and rinsed twice with PTW at room temperature. . Cells were then washed twice with hybridization solution (See Table 4 for composition) twice for 1 hour each at room temperature before incubation in a Techne Hybridiser HB-1D oven at 68^{o}C for 3 hours. The hybridization mix was then replaced with the appropriate pre-warmed probe in hybridization mix and left to incubate in the oven at 68^{o}C overnight.

Table 4: Composition of the hybridization solution

Component (stock conc.)	Final conc.	volume to add
Formamide	50%	25 ml
SSC (20x, pH 5.3 adjusted with citric acid)	1.3x SSC	3.25 ml
EDTA (0.5M, pH 8.0)	5mM	0.5 ml
Yeast RNA (20mg/ml)	50µg/ml	125 µl
Tween-20	0.002	100 µl
CHAPS (10%)	0.005	2.5 ml
Heparin (50 mg/ml)	100µg/ml	100 µl
H_2O		~18.4 ml
Total:		50 ml

On the second day, the cells were rinsed once and washed twice (30 min each) in pre-warmed hybridization solution, then a further 20 min in 1:1 hybridization solution: TBST (composition?) at 68^{o}C. Followed by 3 1-hour washes in TBST at room temperature. Cells were then incubated in blocking buffer (TBST containing 5% heat inactivated sheep serum and 1 mg/ml BSA) for 1 hour before incubation overnight at 4 ^{o}C in a 1:5,000 dilution of alkaline-phosphatase-conjugated anti-DIG antibody (manufacturer?) in blocking buffer.

On the third day, cells were rinsed three times and then washed three times (one hour each) in TBST. After two 10 min washes in NTMT (composition?), alkaline phosphatase activity was revealed by incubation at room temperature

in NTMT containing 4.5 µl nitro-blue Tetrazolium (NBT; 75mg/ml in 70% DMF) and 3.5 µl bromo-chloro-indole phosphate (BCIP; 50mg/ml in 100% DMF) per 1.5 ml. Staining required between 15 min and occasionally up to 48 hours at room temperature. The staining reaction was then stopped by washing twice for 10 min in TBST. Cells characteristics were recorded photographed and fixed as in the procedure described above.

2.9. Immunohistochemistry and β-gal staining of chick embryonic stem cells (cESCs).

2.9.1. β-Gal staining

Chick embryonic stem cells were fixed with freshly made 4% formaldehyde in PBS for 15 minutes. They were then rinsed with PBS twice before incubation in X-Gal solution (9 ml water, 500µl PBS x20, 200 µl $K_4[Fe(CN)_6]$, 200 µl $K_3[Fe(CN)_6]$, 20 µl $MgCl_2$ and 100-200 µl freshly thawed X-Gal at 37°C for between 1 hour and overnight, as required. The reaction was stopped with PBS or PBT.

2.9.2. Immunohistochemistry and staining with DAPI

To reveal GFP from reporter plasmid transfections, or assess molecular cell characteristics of cES cells the following immunological methods were used. Chick ESCs were fixed in 4% paraformaldehyde in PBS for 15 minutes at 4°C, washed in PBS three times for 5 minutes and blocked in 1% Triton-X100 and 1% BSA in PBS for 30 minutes at room temperature. The primary antibody (diluted in blocking buffer) was then added overnight at 4°C. The cells were then washed with PBS (3 x 5 minutes), and then incubated overnight at 4°C in the secondary antibody (diluted in blocking buffer). The cells were then washed in PBS (3 x 5 minutes) and mounted with a glass coverslips in (Citiflour) mounting medium.

In some experiments, following the washes and before mounting, cells nuclei were stained with DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) (Roche Diagnostics , Germany) (Russel et al., 1975) [Diluted in distilled water to constitute 5mg/ml stock solution and in methanol to a final concentration of 1µg/ml to constitute working solution] using the following steps: First, cells were rinsed once with DAPI-methanol working solution. Secondly, cells were incubated in DAPI-methanol at 37ºC. for 15 minutes. Thirdly, DAPI-methanol solution was removed. Finally, the cells were rinsed once with absolute methanol, air dried, then mounted in coverslips of stored in fix as described above.

2.9.3. Antibodies

Primary antibodies for 3A10 neurofilament (post-mitotic neuron specific marker) (Furley et al., 1990; Storey et al., 1992) and the glial cell marker EAP3 (McCabe et al., 1992; Napeir et al., 1999; Yuan et al., 1997) were bought from the Developmental Studies Hybridoma Bank (Iowa University, USA). TUJ-I: A neuron specific (Ferreira and Caceres, 1992; Maurer et al., 2007; Miura and Kameda, 2001; Scott et al., 1990) beta III tubulin antibody was bought from Abcam, (UK). Secondary antibodies (Alexa Flour 488 (green),

Table 5 provide the details of the main antibodies used in this project and their characteristics.

Table 5: Antibodies Description

Antibody	Antigen	Source	Cells/ Flourophore detection colour	Species	Host	Type
Primary	3A10 (Neuro-filament)	DHB§ USA	Postmitotic neurons	Chick	Mouse	IgG1
Primary	EAP3 (Transitin)	DHB USA	Glial cells	Chick	Mouse	IgG
Primary	TUJ-1 (Class III β tubulin)	Abcam UK	Neuron specific	Chick	Mouse	IgG2a
Primary	GFP	Invitrogen UK	GFP	Rabbit	Rabbit	IgG
Secondary	Mouse IgG (H+L)	Invitrogen UK	Alexa Flour (Red)	Mouse	Donkey	αIgG
Secondary	Mouse IgG (H+L)	Invitrogen UK	Alexa Flour 488 (Green)	Mouse	Donkey	αIgG
Secondary	Rabbit IgG (H+L)	Sigma UK	Alexa Flour (Red)	Rabbit	Donkey	αIgG

§ DHB: Developmental Studies Hybridoma Bank (Iowa University, USA)

Chapter 3. Identification of enhancers responsible for Sox2 expression in chick ES cells

3.1. Introduction

As discussed in Chapter 1 Sox2 is a definitive neural marker (Albazerchi and Stern, 2007; Linker et al., 2009; Papanayotou et al., 2008; Stern, 2006) which plays a key role in regulating the factors implied in specification of early neural tissue and subsequent development of the central nervous system of the vertebrates' embryos (Catena et al., 2004; Cavallaro et al., 2008; Ellis et al., 2004; Episkopou, 2005).

Cross-species comparisons of non-coding regions associated with the Sox2 locus identified 25 highly conserved blocks of non-coding sequences which were then tested using reporter assays (Uchikawa et al., 2003). Although the expression of *Sox2* mRNA in the developing chick embryo from stage 4 (Hamburger and Hamilton, 1951) seems uniform throughout the neural plate and consequent developing CNS, the above study revealed that this pattern of expression results from the sum of the activities of five distinct enhancers (N1 through N5) which are found widely scattered upstream and downstream of the sole exon of Sox2. Reporter assays revealed that these five enhancers not only have distinct spatial expression but are also activated at different times: N-2 is activated first, followed by N-1 and finally N-3, N-4 and N-5. Five other enhancers controls its expression in other locations of the developing embryo leaving fifteen conserved sequences with no identified activity (Kamachi et al., 2009; Kondoh and Uchikawa, 2008; Uchikawa et al., 2003)

Work on regulation of Sox2 in early embryo CNS development in other animal models showed that as it is in the chick developing embryo, the expression of Sox2 mRNA during other vertebrates' embryo neural development as well as

in mouse embryonic and neural stem cells is regulated by these very enhancers. (Catena et al., 2004; Inoue et al., 2007; Kamachi et al., 2009; Zappone et al., 2000)

The experiments in this Chapter were designed to test whether any of the 25 enhancer elements described by H. Kondoh's group as being responsible for regulating Sox2 expression in the embryo can also drive expression in proliferating chick embryonic stem cells.

3.2. Methods

Three cell lines of chick embryonic stem cells were maintained and expanded in 48 well plates as described in Chapter 2. Mitomycin C treated STO feeder cells seeded at confluence concentration were used as a negative control. For each cell line two parallel independent experiments were performed. In each experiment plasmids containing one of 25 Sox2 conserved regulatory elements [Total of 26 plasmids containing all 25 conserved sequences (except sequence sumber 10) with two conserved sequences (No. 13, 22) having two corresponding plasmids each] reporter plasmids as well as two other plasmids (pCAβ-gfp and tk-EGFP) were used. The pCAβ-GFP plasmid controls for transfection efficiency (positive control) and the tk-EGFP plasmid controls for the baseline activity of the minimal promoter thymidine kinase when it is not coupled to an identified regulatory element (negative control). Cells were transfected with plasmid DNA with Lipofectamine^{TM} 2000 as described in Chapter 2.

The activity of the regulatory element being tested was assessed by percentage of green fluorescent cells to the total of cells observed 36 hours following transfection. Six consecutive 20x objective fields were scored for each well (experiment). Fluorescent cells were counted after 36 hours of transfection. Cell counts were logged into a spread sheet and the percentages of green fluorescent cells over total number of cells in the field were

calculated. For each experiment descriptive statistics were calculated using SPSS programme and comparison of plasmid activity was made by non-parametric Kruskal-Wallis test. A P value of less than 0.05 was accepted as statistically significant. Planned Mann-Whitney follow up tests with Bonferroni correction were used to compare the difference of plasmid activity between the three cESC cell lines and the feeder layer cells.

3.3. Results

3.3.1. Activity of Sox2 conserved sequence blocks in cES cell lines and STO feeder cells

A detailed record of activity of different plasmids used in this experiment in the four cell lines transfected is presented in Table 6. Figure 3.1 represent a graphical presentation of a summary of the same data. As the cell counts' distribution was not normal, figures presented include both mean and median percentage of green fluorescent cells with non-parametric tests used for comparisons.

1] The 9N2 cell line

Median transfection efficiency was 61.4% with a range of 38.9 – 73.3%. There was a minimal baseline activity of the thymidine kinase minimal promoter (tk) with 0 – 0.3% of cells scored as positive for the reporter gene GFP. Nevertheless, the median percentage of green fluorescent cells was 0%. Out of the 26 plasmids containing the putative regulatory elements of the sox2 locus only 3 displayed activity with a median green fluorescent cell counts that was greater than the median of the negative control. These three plasmids are C8, C11, C17 with median percentage green cell counts of 19.6%, 0.2% and 2% respectively. Fluorescent were cells observed in wells transfected with plasmid C1, C22.5 and C23 with mean percentage of 0.08, m0.25% and 0.36% respectively. The median percentage of green cells detected following transfection by these two cells was 0% for both of them. No green fluorescent cells were detected following transfection with the other plasmids.

Table 6: The activity of the identified enhancers of Sox2 gene in chick embryonic stem cells

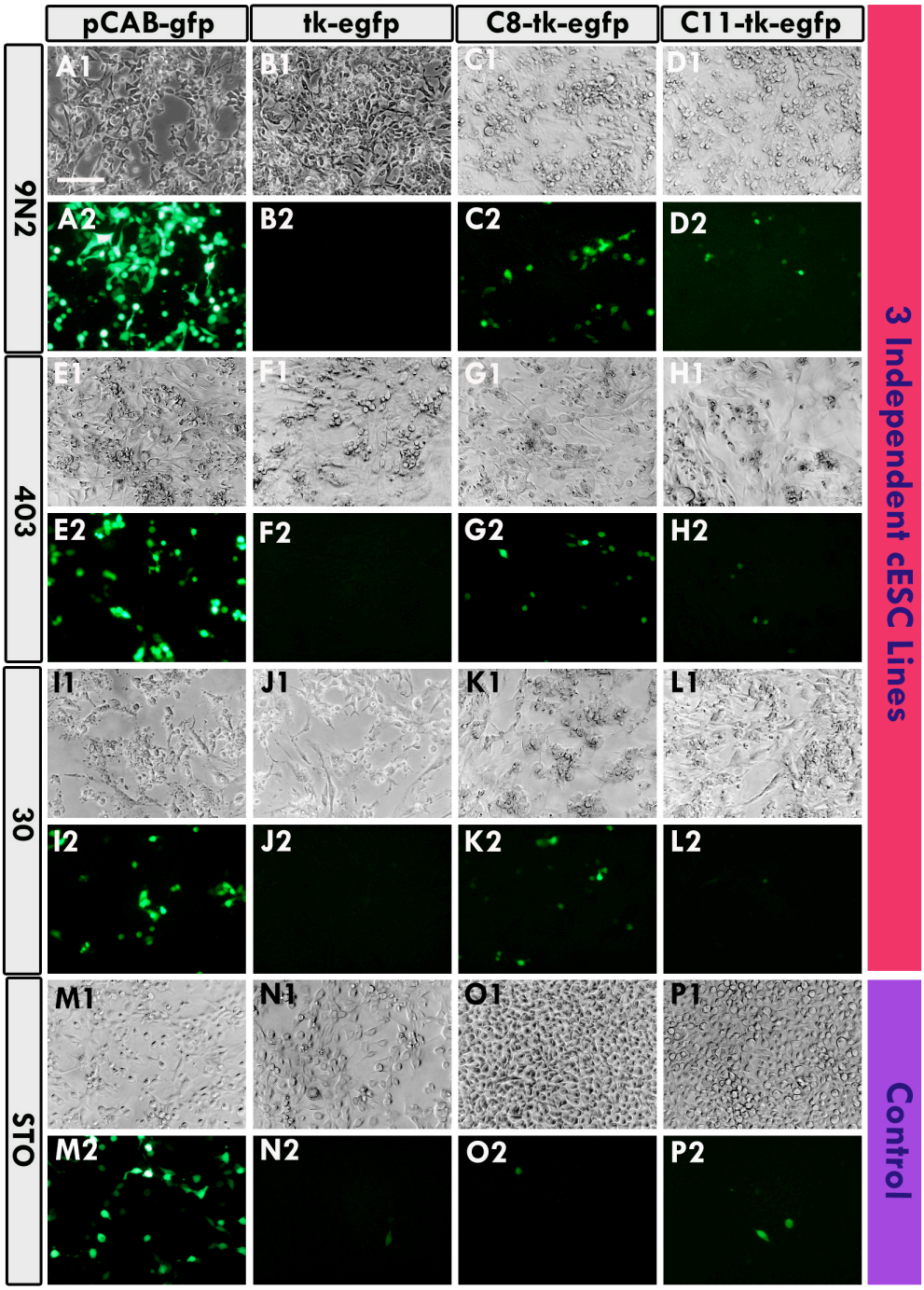
LINE / Plasmid	9N2 cell line			403 cell line			30 cell line			STO feeder cells		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
PCAB	60.1*	9.7	61.4	57.0	7.7	56.6	33.0	10.2	33.5	40.0	8.9	40.2
Tk.GFP	0.1	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.2	0.0
C1	0.1	0.1	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0
C2	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.0	0.0	0.0
C3	0.0	0.0	0.0	0.1	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
C5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C6	0.0	0.0	0.0	0.1	0.2	0.0	0.2	0.8	0.0	0.0	0.0	0.0
C7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C8§	20.0	4.7	19.6	13.4	5.7	11.5	14.0	7.9	13.5	0.0	0.0	0.0
C9	0.0	0.0	0.0	0.1	0.2	0.0	0.0	0.0	0.0	0.1	0.2	0.0
C11§	0.5	0.6	0.2	0.5	0.6	0.3	0.1	0.2	0.0	0.0	0.0	0.0
C12	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.2	0.0	0.0	0.1	0.0
C13	0.0	0.0	0.0	0.1	0.3	0.0	0.1	0.2	0.0	0.1	0.2	0.0
C13.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0
C14	0.0	0.0	0.0	0.1	0.2	0.0	0.1	0.4	0.0	0.0	0.0	0.0
C15	0.0	0.0	0.0	0.1	0.2	0.0	0.0	0.1	0.0	0.0	0.0	0.0
C16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
C17§	1.5	0.8	2.0	1.4	1.6	1.0	0.1	0.2	0.0	0.0	0.1	0.0
C18	0.0	0.0	0.0	0.1	0.2	0.0	0.1	0.1	0.0	0.0	0.0	0.0
C19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
C20	0.0	0.0	0.0	0.1	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C21	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.6	0.0	0.0	0.1	0.0
C22	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.3	0.0	0.0	0.0	0.0
C22.5	0.3	0.4	0.0	0.2	0.3	0.0	0.2	0.3	0.0	0.2	0.3	0.1
C23	0.4	0.5	0.0	0.3	0.5	0.0	0.3	0.6	0.0	0.1	0.3	0.0
C24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C25	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.5	0.0	0.0	0.0	0.0

* Average percentage of fluorescent cells is represented by the mean, SD and median percentages of fluorescent cells in 6 counts of 20x objective field in two independent experiments (wells).

§ Plasmids with conserved sequence which has significant variance of activity in cES cells and STO feeder cells (Kruskal-Wallis test; significance accepted at p value <0.05)

Figure 3.1: Functional analysis of Sox2 regulatory blocks in proliferating chick embryonic stem cells. Transfection with Lipofectamine® in three cESC lines 9N2 (A1 – D2); 403 (E1 – H2); 30 (I1 – L2) and STO feeder layer cells (M1 – P2). The first column shows the results of transfection of the pCAB-gfp plasmid. Green fluorescent cells were detected in all 4 cell lines (A2, E2, I2, M2). The second column shows the baseline activity of the minimal promoter thymidine kinase (tk) with no additional regulatory element.This result matched those of the regulatory elements with no activity in these cell lines under the experimental conditions. The third column shows the results of transfection of plasmid C8-tk-EGFP, corresponding to the N2 enhancer. Green cells were observed primarily in the cESC lines (C2, G2, K2) compared to the control feeder cells (O2). The last column shows the results following transfection with the C11-tk-EGFP plasmid. Green cells were observed mainly in the 9N2 & 403 cESC lines (D2 & H2) where the regulatory element C11 seems to be more active. In the cESC primary line 30 C11 was found to have less activity (L2). Note that C11 also has some activity in feeder layer of STO cells (P2); to a lesser extent other constructs also show including the minimal tk promoter alone (panels N2 and O2, each containing a single, very faintly fluorescent cell [arrows]). Only The N2 enhancer is exclusively active in all cESC lines compared to the feeder layer cells (C2, G2, K2, O2) (Scale bar = 80μm).

Figure 3.1: Functional analysis of Sox2 regulatory blocks in proliferating chick embryonic stem cells.



2] The 403 cell line

Median transfection efficiency was 56.6% with a range of 40.9 – 69%. There was a minimal baseline activity of the thymidine kinase minumal promoter (tk) with 0 – 0.3% of cells scored as positive for the reporter gene GFP. Nevertheless, the median percentage of green fluorescent cells was 0%. Out of the 26 plasmids containing the putative regulatory elements of the sox2 locus the same 3 plasmids found to have activity above the negative control plasmid in the 9N2 cell line were found to have similar activity in the 403 cell line too. Median green fluorescent cell counts percentages for these three plasmids (C8, C11, C17) was 11.5%, 0.25% and 1% respectively. Green fluorescent protein positive cells were also observed in wells transfected with plasmid C1, C3, C5, C6, C9, C12, C13, C14, C15, C18, C20, C22.5, C23 and C25. Only C22.5 and C23 had a mean percentage greater than 0.1% (0.24% and 0.3% respectively). The median percentage of green cells detected following transfection by these two plasmids (as well as the rest of the 26 vectors) was 0%.

3] The 30 cell line

Transfection efficiency was the lowest with median of 33.5 %, range of 11 - 48%. There was no baseline activity detected of the thymidine kinase minimal promoter (tk). Green fluorescent cells were detected following transfection of plasmids C2, C4, C6, C8, C11, C12, C13, C13.5, C14, C15, C17, C18, C21, C22, C22.5, C23, C24, C25. The C8 plasmid had the greatest activity with a median percentage of green cells of 13.5. The activity of the remaining regulatory elements was minimal with a green cells percentage median of 0%.

4] The STO cell line

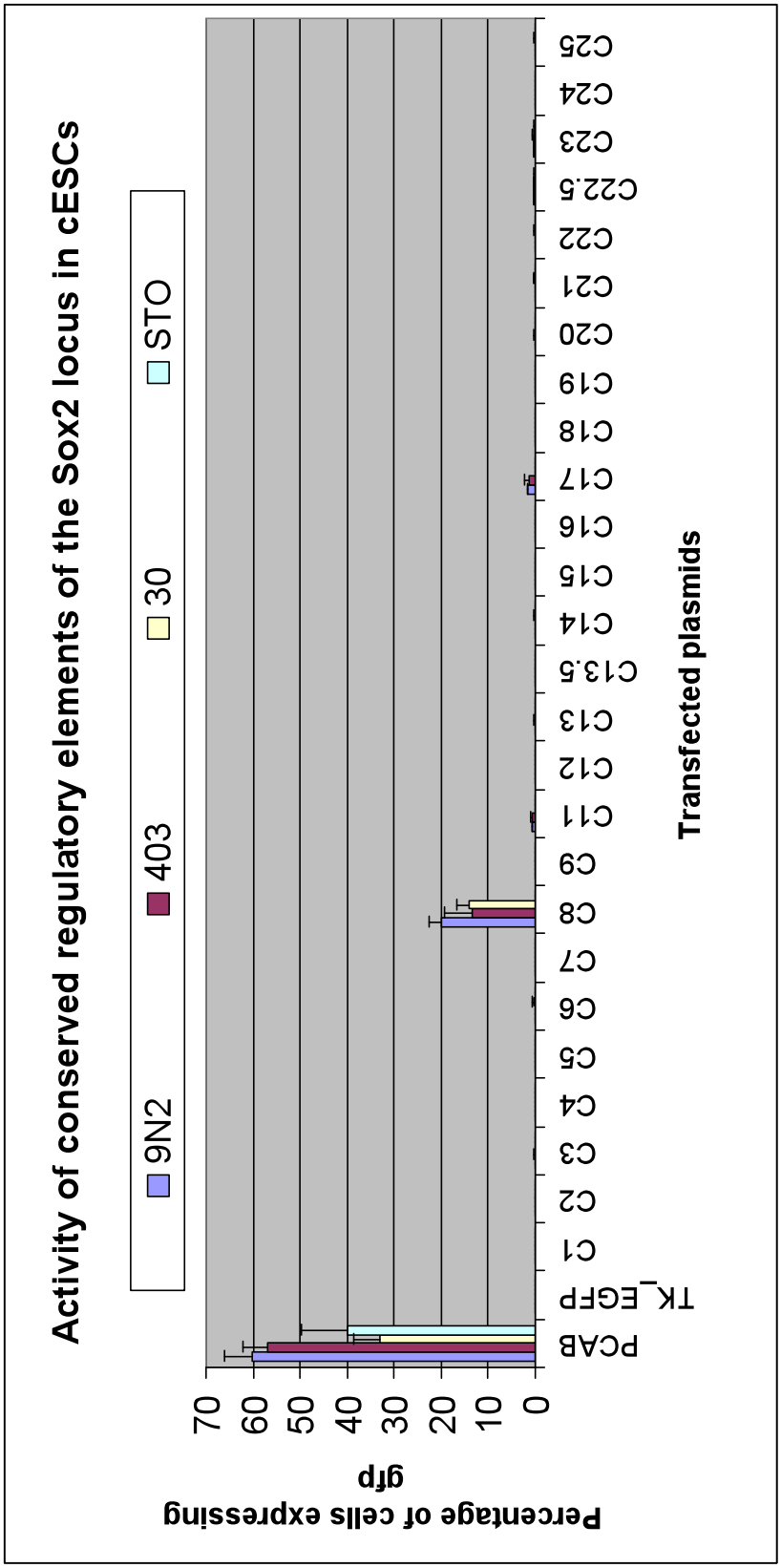
Median transfection efficiency was 47.9 %, range 23.9 - 52%. There was minimal baseline activity detected of the thymidine kinase minimal promoter (tk) with mean percentage green fluorescent cells of 0.08%. Green fluorescent cells were detected in small numbers following the transfection of plasmids

C1, C9, C12, C13, C13.5, C16, C17, C19, C21, C22.5, C23. All such activity was The C22.5, C23 plasmids had the greatest activity with a mean percentage of green cells of 0.17% and 0.13% respectively. The activity of all the remaining regulatory elements was minimal with a green cells percentage median of 0%.

3.3.2. Statistical analysis of activity of Sox2 conserved sequence blocks

Using the Kruskal-Wallis test, the median percentage of green fluorescent cells was found to be significantly different following the transfection of the following plasmids: PCAB-GFP, C8, C11 and C17; $H(3) = 30.2, 31.2, 12.8, 26$ respectively ($p<0.05$). Figure 3.2 shows the average (mean) percentage of green fluorescent cells detected following transfection of all 28 plasmids in the 3 cESC lines as well as in the STO feeder layer cells. The largest proportion of green cells was detected following transfection of pCAB-gfp. Transfection efficiency varied between experiments and cell lines with a mean total efficiency of 47.5 ± 14.5%. Transfection efficiency of the 9N2, 403, 30 cESC lines were 60 ± 9.7%, 57 ± 7.7% and 33 ± 10.2 % respectively. The transfection efficiency of the STO feeder cells was 40 ± 8.9 %. In contrast, a baseline activity of the minimal promoter reporter (tk-EGFP) plasmid was detected in the 4 cell lines. The mean green fluorescent cells detected following tk-efgp transfection in all wells was 0.04 ± 0.12 %.

Figure 3.2: Differences in activity of different plasmids in different cESC lines (9N2, 403, 30) and STO feeder cells



Four different patterns emerged from comparison of the proportion of fluorescent cells following the transfection of the 28 plasmids. First, plasmids with activity in 4 cell lines. Apart from pCAB-gfp (positive control), only C22.5 and C23 had such a pattern, with a few green cells detected in some but not all wells. Overall, the mean proportion of green fluorescent cells detected following the transfection of these two plasmids was 0.2 ± 0.3 % for C22.5 and 0.3 ± 0.5 % for C23.

The second type of pattern observed corresponds to plasmids producing no activity in any cell line. In addition to the negative control plasmid tk-EGFP, most of the 26 constructs yielded this result. Plasmids with sequence blocks C1, C2, C4, C5, C7, C9, C12, C13.5, C15, C16, C18, C19 and C24 had no detectable activity. In addition, plasmids with sequence blocks C3, C6, C13, C14, C20, C21, C22 and C25 can be included in this group as the number of green fluorescent cells detected in some of the wells was similar to that of the negative control tk-EGFP.

The third pattern consists of plasmids with activity in some cESC lines. Plasmids containing C11 and C17 comprise this group. Green fluorescent cells were detected with these two plasmids predominantly in the 9N2 and 403 cESC lines. The former plasmid (C11) produced 0.5 ± 0.6 %, 0.5 ± 0.6, 0.05 ± 0.2 and 0.0 ± 0.0 % respectively in cell lines 9N2, 403, 30, STO lines, and the latter (C17) yielded 1.5 ± 0.8, 1.4 ± 1.6, 0.08 ± 0.2 and 0.01 ± 0.05 % respectively in the same lines.

The fourth pattern was yielded by plasmids showing activity in all cESC lines but not in STO feeder cells. Only C8, corresponding to the N2 enhancer, was in this group. The average proportions of green fluorescent cells detected in the 9N2, 403, 30 cESC lines were 20 ± 4.7, 13.4 ± 5.7 and 14 ± 7.9 % respectively. No activity was detected in the STO feeder layer cells.

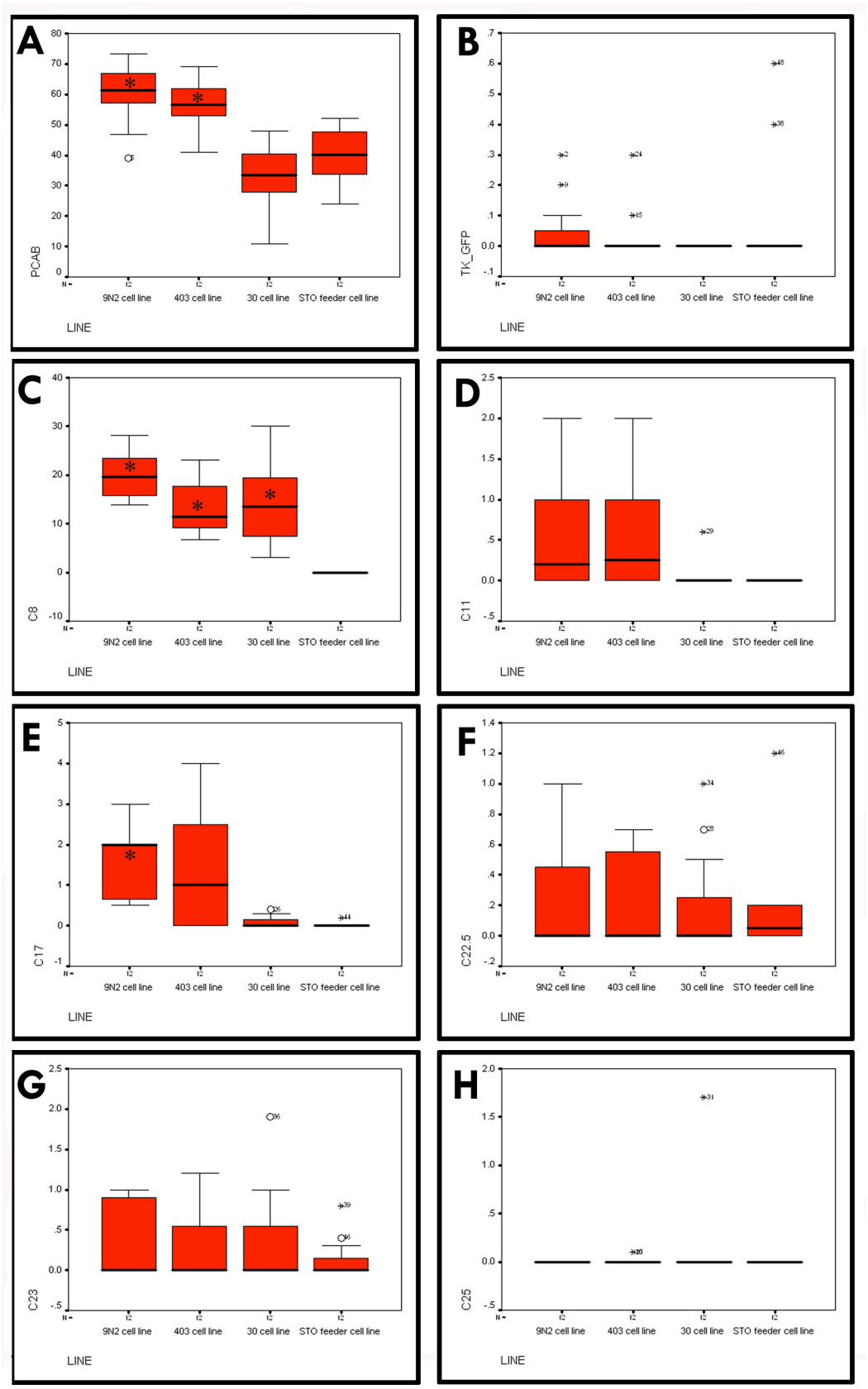
Mann-Whitney tests were used to follow up these findings to compare the percentage of green fluorescent cells between the STO feeder layer and each

of the three cESC lines (3 tests per plasmid). A Bonferroni correction was applied and so statistical significance is only accepted at p value of less than 0.0167.

No significant difference was seen in the percentage of green cells between the STO feeder cells and each of the three cESCs following transfection of the negative control (tk-gfp), C11, C22.5, C23 and C25. The median percentage of green fluorescent cells in the cESC line 9N2 was significantly greater than that of the STO feeder cells following transfection of plasmids pCAβ-GFP, C8, C17. The median percentage of green fluorescent cells observed in the 403 cESC line was greater than that of the STO feeder layer following transfection of pCAβ-GFP and C8 plasmids. Finally, only following transfection of C8 was a significant increase observed in the percentage of green fluorescent cells detected in the cESC line 30 compared to the feeder layer. Thus, although transfection efficiency was greater in cESC lines 9N2 and 403 than in feeder cells, only plasmid C8 (N2 enhancer) had significantly more activity in all cESC lines as compared to that observed in the STO feeder cells. Figure 3.3summarizes these findings.

Figure 3.3: Differences in the activity of plasmids between STO and cESC lines. Box-plot graphs comparing the percentages of green fluorescent cells detected following the transfection of the main active plasmids found. **A.** pCAβ-gfp (transfection efficiency/positive control) activity varied between cell lines and both 9N2, 403 cESC lines displayed greater transfection efficiency than the feeder cells (STO). A significant difference in activity of a conserved *Sox*2 sequence (enhancer) between cES cell lines and STO feeder cells is marked by an asterisk (as a Bonferroni correction was applied, statistical significance is only accepted at p value<0.0167). **B.** Tk-egfp (negative control) showed minimal activity in all cell lines and no difference between feeder and cESC lines. **C**. C8 (N2 enhancer) was the only plasmid with activity exclusively in cESC lines. **D**. Although C11 has activity in 9N2 and 403 cESC lines, this activity was not significantly higher than its activity in the STO cells. **E**. C17 (N1 enhancer) has activity in 9N2 and 403 cESC lines similar to that of C11. The activity of the N1 enhancer, although low, was significantly greater in the 9N2 cESC line than in the STO feeder cells. **F, G.** The C22.5 and C23 elements produce some activity in all cESC lines as well as in the feeder cells but no significant difference is detected between them. **H**. C25 produces very low activity, seen in only two of 48 fields scored. This is representative of what is seen with the remaining plasmids have no activity in either cESC cell lines or in the STO feeder cells.

Figure 3.3: Differences in the activity of plasmids between STO and cESC lines.



3.4. Discussion

By comparing the chick Sox2 locus with that of the mouse and the human, Hisato Kondoh's group identified twenty-five highly conserved sequence blocks (Uchikawa et al., 2003). Electroporation of reporters comprising each of these enhancers along with a minimal promoter (tk) into developing chick embryos was then used to test the domains of the embryo in which each of these elements can drive transcription. Expression of these elements was first detected at around stage 5 (Hamburger and Hamilton, 1951). Within the developing nervous system five enhancers account for the expression pattern of Sox2 mRNA during the first 2 days of development.

N2 is activated earliest and in the largest domain, corresponding to the entire anterior neural plate down to the level of the node at stage 5 (future forebrain, midbrain and anterior hindbrain). Very shortly afterwards, N1 is activated in small triangular regions of paranodal epiblast. This position corresponds to the ''stem zone'' defined by Kate Storey's group (Delfino-Machín et al., 2005). This region contains precursor cells that contribute to both the posterior neural plate (from the posterior hindbrain down to the caudal tip of the spinal cord) and part of the paraxial mesoderm (Delfino-Machín et al., 2005).

Until very recently it was thought that *Sox2* is not expressed in chick embryos before about stage 5 (Rex et al., 1994; Rex et al., 1997b; Uchikawa et al., 2003; Uwanogho et al., 1995) and its expression in chick ES cells was also unknown. However, *Sox2* is expressed in both mouse ES cells (Avilion et al., 2003; Masui et al., 2007) and in the epiblast of the early mouse embryo (Wood and Episkopou, 1999; Zappone et al., 2000). The activity of regulatory elements of Sox2 in mouse embryonic stem cells has been studied by (Zappone et al., 2000) who found activity of a 5' region of about 400 base pairs when linked to a 3.3 kb region upstream of the Sox2 reading frame which they consider to contain a Sox2 minimal promoter (Catena et al., 2004;

Zappone et al., 2000). Sequence comparisons reveal that the former 400 base pair region corresponds to the chick N2 enhancer of (Uchikawa et al., 2003). The work presented in this chapter suggests that out of the 25 elements identified by Kondoh, the N2 enhancer is the one that most strongly drives expression of a GFP reporter in chick embryonic stem cells. The activity of this enhancer is also specific to cES cells as no significant activity was seen in the STO feeder cells.

Our work utilized the minimal promoter thymidine kinase rather than the putative Sox2 minimal promoter identified by Catena et al. (2004). The very low baseline activity of the tk promoter enables the activity of the N2 enhancer to be determined more easily and reveals that N2 is sufficient to drive expression in cES cells independently from the 3.3 kb segment upstream of the *Sox2* open reading frame (Catena et al., 2004).

It is also worth noting that only 20% of cells appear to display activity of the N2 enhancer. *Sox2* mRNA expression can be detected in up to 90% of cESCs (see below Chapter 5). Limited transfection efficiency is probably not sufficient to account for this difference because even in the 9N2 cell line which displays the highest levels of N2 activity, transfection efficiency (measured by activity of the ubiquitously-expressed pCAβ-EGFP reporter) is 60%. Ideally, curves with increasing amount of DNA should have been constructed to maximise the transfection efficiency for each of these plasmids, and this should have been coupled with co-transfection of a ubiquitously expressed reporter of transfection efficiency producing fluorescence of a different colour (eg. DS-Red or RFP). Differences in the efficiency of the different promoters, DNA concentration (including those due to differences in plasmid length) and other factors could contribute to account for the differences. In addition, it is possible that elements in addition to N2 contribute to expression in cES cells and could account for the difference between the 20% observed and the expected 54% (90% adjusted for transfection efficiency). These additional elements could be among those identified by (Uchikawa et al., 2003) and also tested here, if they only work in combination, but they could include additional elements not identified from Kondoh's study. Further work, starting from exploring

combinations of the elements tested here, is needed to answer these questions.

In their study of enhancers driving Sox2 expression in mouse ES cells, Catena et al (2004) showed that the mouse enhancer sequence equivalent to the N2 region contains multiple POU binding sites. Using gel shift assays and chromatin immunoprecipitation they showed that Oct1 and Oct4 can bind to these domains both in vitro and vivo (Catena et al., 2004). The chick Oct3/4 homologue was originally thought not to exist (Soodeen-Karamath and Gibbins, 2001). However more recently PouV has been identified as the chick homologue of mammalian Oct3/4, its expression demonstrated in chick embryonic stem cells along with a role in regulating pluripotency of these cells (Lavial et al., 2007) . PouV may therefore be an important factor in regulating *Sox2* expression in chick ES cells by binding to the N2 enhancer.

The low levels of activity shown by some of the other elements in this study, and especially C11 and C17 (N1 enhancer), might be explained by a low level of spontaneous differentiation. This is consistent given the low number of cells that show these activities as compared to the C8 region (N2 enhancer). It is also possible that C11 and/or C17 are among additional elements required to account for the full expression of *Sox2* in cES cells as discussed above, and these may only work in conjunction with N2 and/or other elements.

The other regions found to have some activity in ES cells also have activity in STO cells: C22.5 and C23. The expression in STO cells (which are not known to express Sox2 mRNA) suggests that this activity is likely to be an artefact, perhaps due to sequestering ubiquitous or other transcription factors expressed in these cells.

The differences in activity of the N2 region and mRNA expression in cES cells could also indicate the existence of silencers within N2 or in other DNA regions. To identify these regions quite different experiments need to be designed, including nested deletions and/or other mutations of the reporter constructs. A final possibility is that the lower expression seen with the N2

reporter constructs as compared with *Sox2* mRNA in cES cells might partly be due to competition of the transfected reporter for transcription factors that are already engaged in activating the endogenous *Sox2* gene. It might be interesting to compare the levels of expression of *Sox2* mRNA in cells transfected with a N2 reporter as compared with other sequences: if this competition is taking place one might find reduced Sox2 mRNA expression in N2-transfected cells.

Chapter 4. Methods for inducing neural differentiation in chick ES cells

4.1. Introduction

Since their initial isolation from mouse embryos (Evans and Kaufman, 1981;Martin, 1981), embryonic stem (ES) cells have offered new tools to explore many aspects of the control of cell commitment and differentiation. Differentiated cell types belonging to descendants of all three germ layers of the embryo can be produced from mammalian ES cells. Examples from the mouse model include endodermal derivatives such as the yolk sac (Doetschman et al., 1985) or the pancreas (Ku et al., 2004); mesodermal tissues: primitive and definitive hematopoietic cells (Nakano et al., 1996) , cardiomyocytes (Doetschman et al., 1985), striated (Rohwedel et al., 1994) and smooth (Yamashita et al., 2000) muscle fibers , adipocytes (Dani et al., 1997), chondrocytes (Kramer et al., 2000) and osteoblasts (Buttery et al., 2001) and ectodermal derivatives such as skin (Bagutti et al., 1996), neuronal (Bain et al., 1995; Strubing et al., 1995) and glial lineages (Brüstle et al., 1999; Fraichard et al., 1995; Liu et al., 2000).

Although ES cells are capable of differentiating into potentially all somatic cell types, the study of the events of cell commitment and of differentiation into each of these fates requires reproducible methods to direct them to specific fates. For in vitro induced neural differentiation of ES cells, three different strategies have been established with variable efficiency and reproducibility: embryoid body (EB) formation-based method (Doetschman et al., 1985), stromal cells-mediated method (Kawazaki et al., 2000) and methods based on differentiation of ES cells as a monolayer in defined medium (Tropepe et al., 2001; Ying et al., 2003; Ying and Smith, 2003).

To date, reproducible protocols have only been described for mouse ES cells although pioneering studies by Pain et al. (1996) demonstrated the ability of chicken ES cells to produce neurons in vitro. In this paper, cESCs were found to have the ability to differentiate into neuron like cells following Embryoid Body formation as well as with spontaneous differentiation. These cells were positive to the neuronal marker N-CAM. No other reports of cES induced neural differentiation exist as their multi-germ layer differentiation potential is explored in vivo (Petitte et al., 2004). None of the cES cells induced differentiation methods provide insights about the efficiency of in vitro induced neural differentiation (Acloque et al., 2001; Lavial and Pain, 2010; Pain et al., 1996; Petitte et al., 2004).

In addition to the lack of reproducible methods for generating neural cells from chick ES cells, no study has yet directly compared these different strategies described above in any system model using the same outcome measure (See Table 7). This chapter compares the effectiveness of two of the three main methods to induce neural differentiation of mouse ES cells for the chick ES cell system.

Table 7: Reports on in vitro induced neural differentiation of embryonic stem cells in mouse and chick

Reference	Model	*In vitro* method	Outcome measure	Efficiency
Bain et al 1995	Mouse	**Embryoid Bodies (EB)** [-/+ RA $5x10^{-7}$M] 14 days	Morphology + RT-PCR (Brn3,GFAP,TH)	38% neuron like cells on day 7
Fraichard et al 1995	Mouse	**EB** 2+/7-/D&P 20 days [-/+RA 10^{-7}M]	Morphology + RT-PCR (Nestin, GFAP, O4,	10% neuron like day 6
Wichterle et al 2002	Mouse	**EB** 3+/5- days [-/+RA 10^{-7} – $2x10^{-6}$ M] **Co-Culture** on **Pa6** for 6 days	Section of EB and IHC (Sox1, Otx2, Hoxc6, NeuN., En1,Tuj1)	40-60% Sox1+ day 3 50-70%NeuN+ day 7
Ying et al 2003	Mouse	**Monolayer** N2B27 medium [+/- Shh/FGF4] up to	ES Cell reporter Cell line, FACS	60% Sox1+ day 5 60%Tau-gfp + day 7
Pain et al 1996	Chick	**Embryoid Bodies** [-/+ RA $5x10^{-7}$M] 15 days	Morphology + IHC (N-CAM)	Not reported
This study	Chick	**EB** [+/- **RA**10^{-6}M] 15 days **Monolayer** with N2B27 [+/-RA $5x10^{-7}$-$5x10^{-6}$M] 21 days	Morphology, IHC (3A10 neurofilament, Transitin, TUJ-1)	28% 3A10+ cells day 21 5% Transitin+ cells day21 30+% 3A10+ day 21 10%+ TUJ-1+ day 21

4.2. Methods

4.2.1. Proliferating cES cells expansion

BRL conditioned medium supplemented with supplemented with: 1 mM sodium pyruvate (Gibco, UK), 1% non-essential amino acids (Gibco,UK), 1% Vitamins (Gibco, UK), 1 mM of each nucleotide (adenosine, guanidine, cytosine, uridine, thymidine; Chemicon, USA), 0.16 mM b-mercaptoethanol (Chemicon, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, UK) (ESA medium) was used to grow and expand embryonic stem cells in their proliferating multipotent state as discussed in Chapter 2 above.

4.2.2. Development of embryoid bodies in vitro

ES Cells were dissociated, washed in Ca/Mg free PBS and plated in low-adhesion culture dishes (Nunc) at $5x10^6$ cells/ml ESA medium without LIF. Fresh medium was added every 2 days for 4 days. ESA medium was supplemented with 10^{-6}M of all trans-RETINOIC ACID (Product No R2625, Sigma UK) for another 4 days. After 8 days, floating masses of cells were collected, washed carefully, dissociated in trypsin (0.25% trypsin/EDTA in PBS with 1:5 ratio) and plated onto gelatine coated tissue culture wells or glass coverslips to allow them to attach, spread and differentiate.

4.2.3. In Vitro Differentiation on a monolayer

Proliferating cES cells were dissociated and plated onto gelatine coated dishes without feeder cells at a density of $5x10^4/cm^2$ in DMEM or N2B27 medium (Brewer et al., 1993;Ying et al., 2003;Ying and Smith, 2003) supplemented with 0.5% Fetal Bovine Serum. In experiments that had a 2-day pulse of Retinoic acid (see below), all trans-Retinoic acid (Sigma R2625) was added to the medium in the first 2 days in either low ($5x10^{-7}$M) or high concentration ($5x10^{-6}$M). The medium was renewed every 2 days for 20 days. On the 21st day post-treatment, cells were fixed and stained as described below. As a control and for assessment of the incidence of spontaneous

differentiation, cES cells were kept in ESA – complete BRL conditioned medium for the duration of the differentiation protocol.

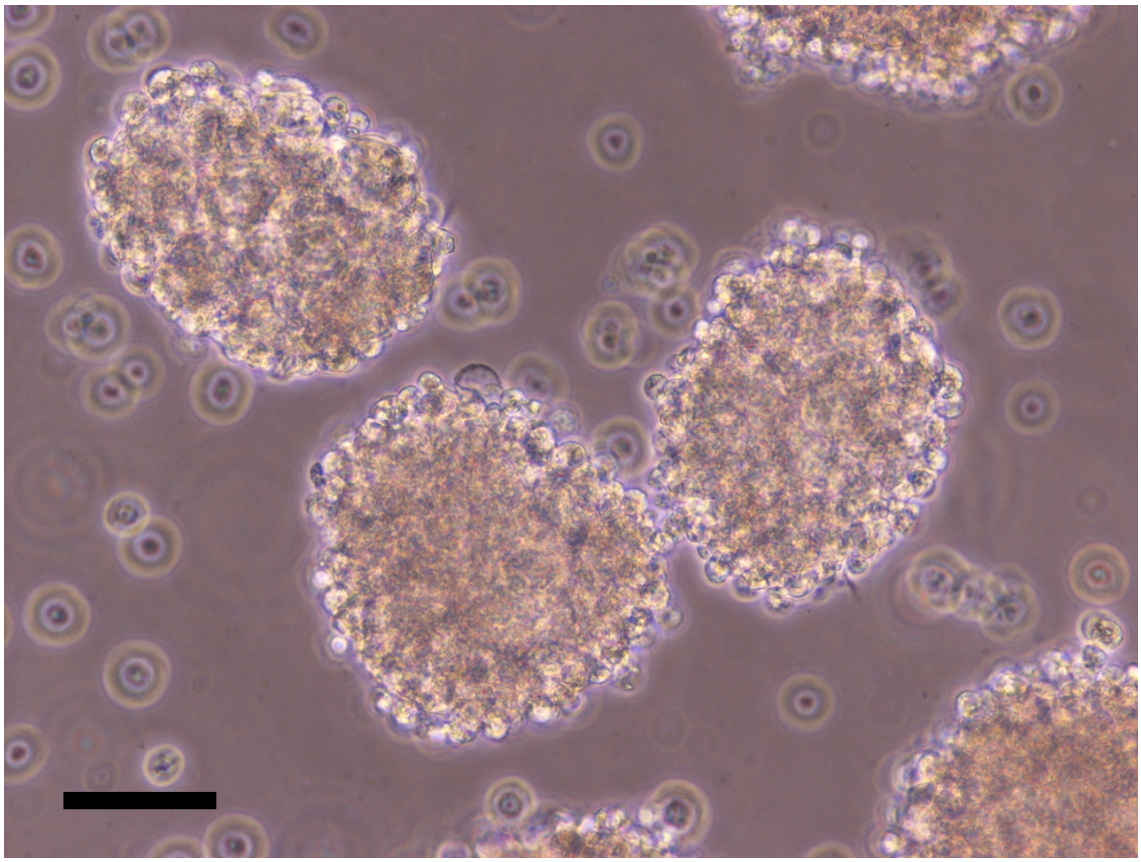
4.3. Results

4.3.1. Embryoid body-based protocol

The purpose of ultra-non adhesive dishes is to prevent cell attachment, which favours the formation of Embryoid Bodies (EB) (Doetschman et al., 1985). Within 18 – 24 hours of plating, cell aggregates appeared in suspended lumps resembling embryoid bodies of mammalian ES cells (Bain et al., 1995). By day 2 these have become large and spherical (See Figure 4.1). On day 5 the medium was changed; half the wells received medium supplemented with 10^6Mall-trans RA.

Figure 4.1: Embryoid Bodies (EB) – like aggregates of cES cells**:** cES Cells aggregates resembling mammalian Embryoid Bodies appear at the end of the first day of passaging multipotent cells in ultra low adhesive tissue culture plate and withdrawing proliferation-maintaining growth factors (Scale bar = 50µm)

Figure 4.1: Embryoid Bodies (EB) – like aggregates of cES cells



On day 9 the aggregates were dissociated with Trypsin EDTA solution and gentle pipetting and plated on regular tissue culture dishes and cultured for a further 4 days in a RA-free medium. Cells were seen to attach to the plates quickly and cells started to spread out from the partly-dissociated EBs, . After 4 days, cells started acquire different morphologies, quite different to that of the starting multipotent cES cells (Figure 4.2). Instead of the spindle-like morphology of cES cells, there were many cells with needle-like shapes (a small body with multiple thin processes), astrocyte-like shapes (star-like, irregular and flat cells with short pointed processes) and larger flat cells which cover almost the entire surface of the culture dish.

To test for neuronal and glial differentiation cells were fixed and stained for markers of differentiating neurons (3A10; neurofilament) (Furley et al., 1990; Storey et al., 1992) and immature neural progenitors/glial cells (EAP3; transitin) (McCabe et al., 1992; Yuan et al., 1997), which is considered to be the chick equivalent of nestin (Napeir et al., 1999). In the RA treated group 11.3% ± 4.1 (S.D.) of cells stained with 3A10. In the control group 2.1 ± 0.7% of cells expressed 3A10. 15.9 ± 1.4% of cells in the RA group were staining for transitin, compared with 4.1 ± 3.3% in the control group. These differences between the RA and control were statistically significant ($p<0.05$). (See Table 8 for summary).

Figure 4.3 (also quantification in Figure 4.4) shows examples of cES cells expressing 3A10 and EAP3. Neither antibody produced staining of proliferating cES cells (see below) and there was also no staining when the primary antibody was ommitted (control not shown). These findings confirm earlier findings that cES cells can be directed to neuronal fates by an embryoid body-mediated method (Pain et al., 1996).

Table 8: Differentiation of cES cells with an EB based protocol.

		4-/4+/D&P 4			4-/4-/D&P 4		
Marker	**Exp**	**Positive cells**	**total cells counted**	**%**	**positive cells**	**total cells counted**	**%**
3A10	1	64	413	15.5%	4	325	1.2%
	2	37	506	7.3%	11	416	2.6%
	3	48	433	11.1%	7	298	2.3%
			Mean	**11.3%**		**Mean**	**2.1%**
			SD	**4.1%**		**SD**	**0.7%**
Transitin	1	123	723	17.0%	12	512	2.3%
	2	88	614	14.3%	28	354	7.9%
	3	91	554	16.4%	9	442	2.0%
			Mean	**15.9%**		**Mean**	**4.1%**
			SD	**1.4%**		**SD**	**3.3%**

Figure 4.2: Changes in cell morphology following induced differentiation of cES cells: A1,2: Chick ES cells have a spindle-like morphology with large prominent nucleus and relatively little cytoplasm. Following induced neural differentiation of cES cells either with EB based protocol or as a monolayer, striking morphological changes occur (**B-D**). The main emerging morphological patters of differentiated cES cells are needle-like (**B1,2**) (a small body with multiple thin processes), astrocyte-like (**C1,2**) (star-like, irregular and flat cells with short pointed processes) and larger flat cells which cover almost the entire surface of the culture dish (**D1,2**). (Scale bar in A1 = 50μm).

Figure 4.2: Changes in cell morphology following induced differentiation of cES cells

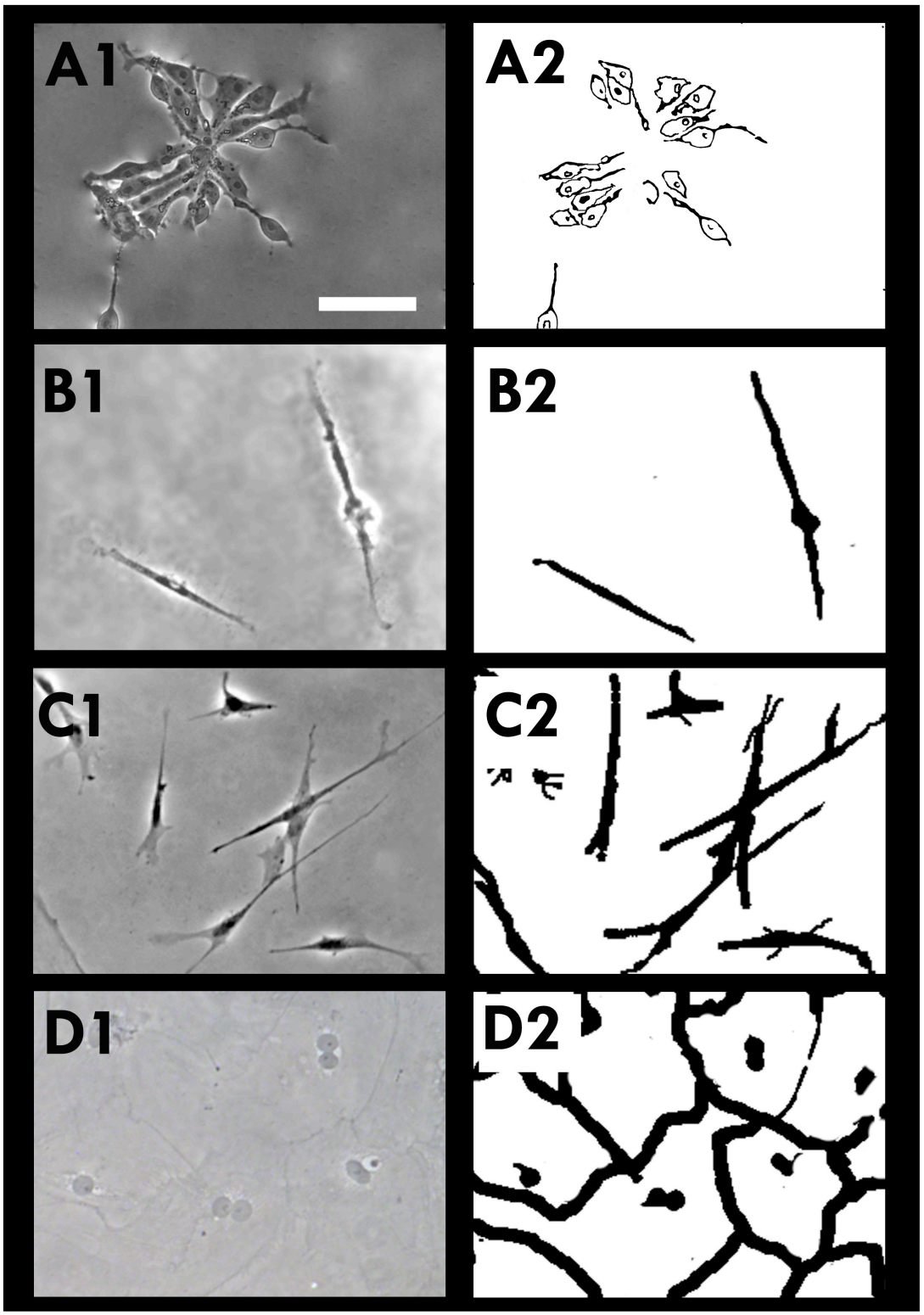


Figure 4.3: Neural marker expression in cES cells from embryoid bodies: A-D: Neuronal (3A10) and glial (EAP3: Transitin) markers were detected following EB formation. More cells expressing these neural markers was noted with addition of Retinoic Acid (A,B) compared with the control (C,D) (See also Figure 4.4) (Scale bar in A1 = 50μm).

Figure 4.3: Neural marker expression in cES cells from embryoid bodies

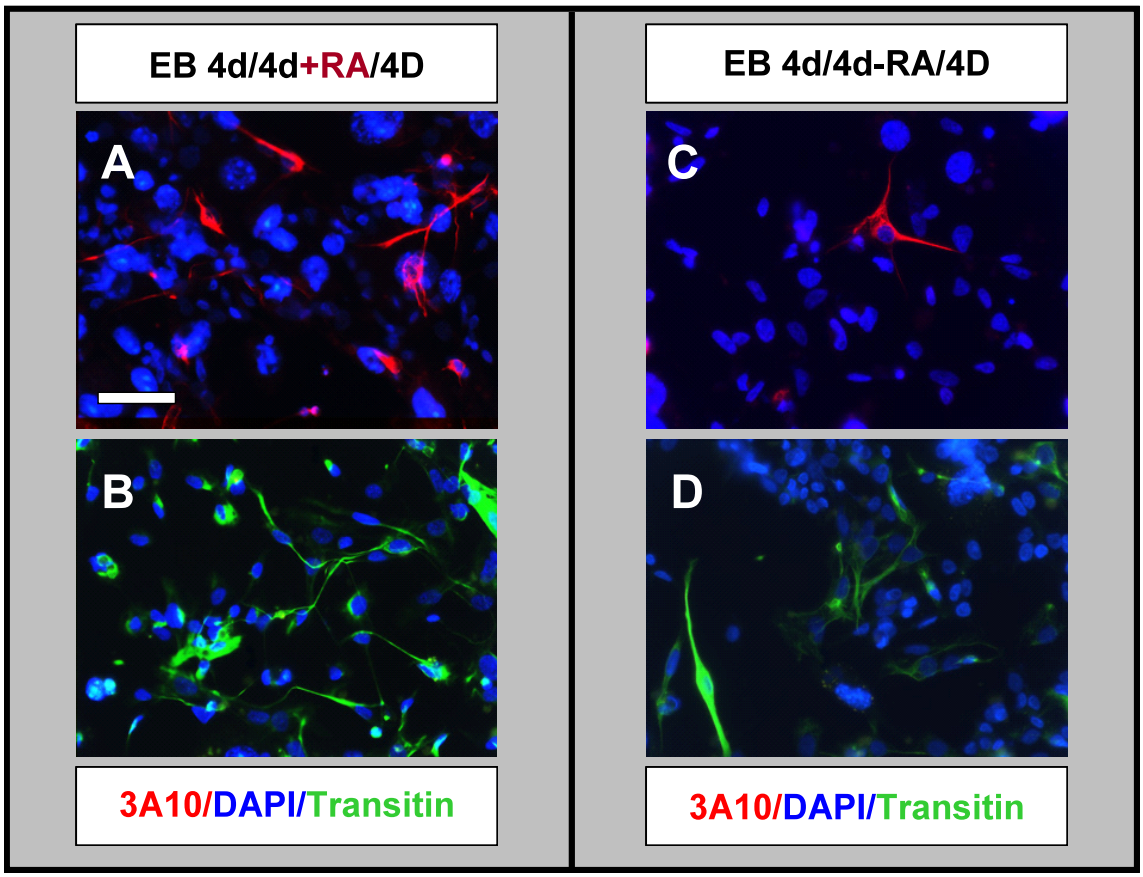
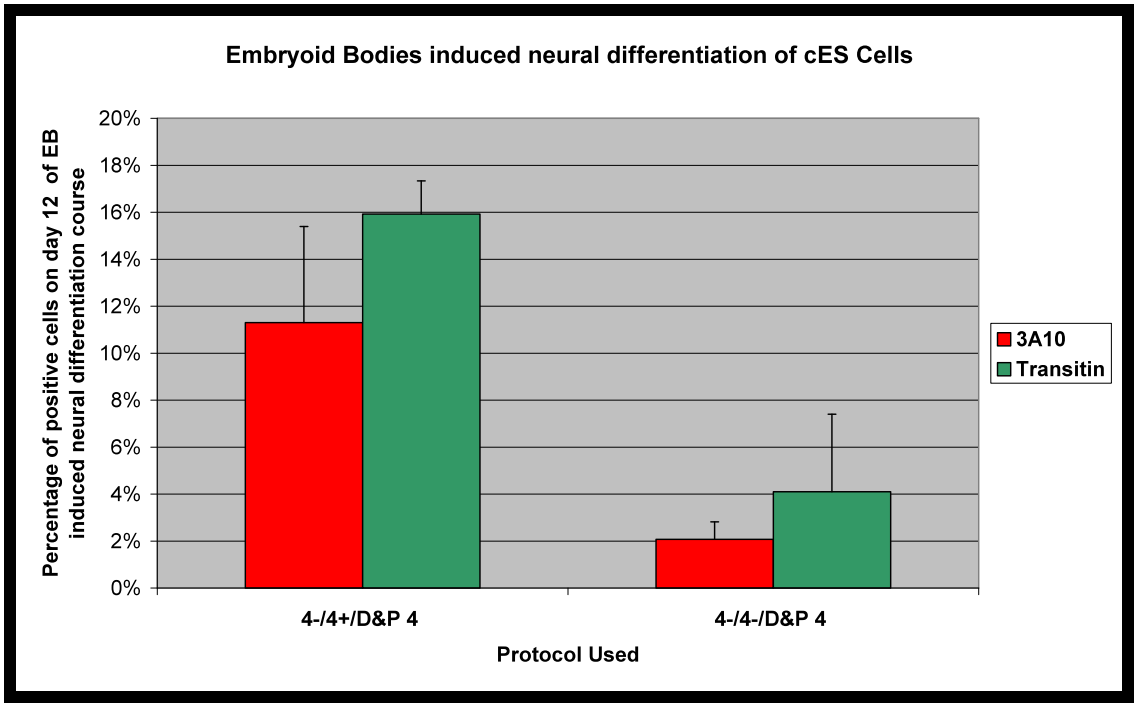


Figure 4.4: Quantification of cES cell differentiation from embryoid bodi: Neuronal (3A10) and glial (EAP3: Transitin) markers were detected following EB-like aggregates formation. More cells expressing these neural markers was noted with addition of Retinoic Acid compared with the control (See also Figure 4.3).

Figure 4.4: Quantification of cES cell differentiation from embryoid bodies



4.3.2. Monolayer culture in defined medium

Neural differentiation of ES cells via embryoid bodies (Bain et al., 1995; Wiles and Johansson, 1999), culture in suspension (Tropepe et al., 2001) or co-culture with stromal cell lines (Kawasaki et al., 2000) have all been criticised as being difficult to control as well as for producing very heterogeneous cultures (Smith, 2001). The use of defined media would eliminate the variability of serum containing medium, improving reproducibility. By developing a reporter cell line in which green fluorescent protein was inserted into the *Sox1* locus, Ying et al at (2003) showed that monolayers of mouse ES cells could be efficiently directed to a neural fate including neuronal and glial cell cell types by modifying the medium in which these cells are cultured (Ying et al., 2003). These authors introduced a medium known as N2B27 which was found to be particularly efficient at supporting neural differentiation (Brewer et al., 1993; Ying et al., 2003; Ying and Smith, 2003).

This method (Ying et al., 2003; Ying and Smith, 2003) was tested for cES. As described for mouse ES cells, in vitro differentiation of cES cells is started by removing factors that maintain their proliferative state (feeder cells, conditioned medium) and exposing them to a two day 'priming' period when cells are exposed to RA followed by growth in N2B27 medium for 18 days (Ying et al., 2003). RA was used at two concentrations: $5x10^{-7}$ M and $5x10^{-6}$ M (represented in figures and tables as +ra and +RA respectively). To assess the effect of N2B27 on efficiency of induced neural differentiation, control DMEM was used in both the initial priming and subsequent 18 days differentiation periods. On the 21st day cells were fixed and stained for EAP3 and 3A10 markers.

Chick ES cells do not express 3A10 or EAP3 when cultured in standard cES medium (where they continue to proliferate) (Figure 4.5: A1-4). On the other hand, many cells with neuronal morphology and 3A10 and EAP3 expression

are seen following the 20 days treatment with N2B27, both with and without RA (Figure 4.5 and Figure 4.6).

Figure 4.7 and Table 9 present a summary of the results of different combinations of the DMEM, N2B27 media with or without RA. The lowest level of differentiation for both markers was seen when cells were primed and cultured in plain DMEM medium. This medium failed to support cell proliferation as compared cells grown using both feeder layers and ESA (Complete BRL conditioned medium). Only a small number of cells survived the former culture conditions (Figure 4.5; compare [A1-4] and [B1-4]). The highest amount of neuronal differentiation was seen when cells were grown in N2B27 following priming with the higher concentration of RA. This produced cultures containing many neurite processes forming complex networks (Figure 4.6 I1-4). Other conditions produced neuronal-like cells with variable efficiency (Figure 4.5 and Figure 4.6 : C1-4 through H1-4; Figure 4.7 and Table 9).

Neurites seemed to extend from areas of greater cell density (Figure 4.5: D1,D2; Figure 4.6: F1,F2; G1,G2 and I1,I2]. The complexity of neurite networks also varied between conditions. To evaluate the networks a 4 point score was devised. The presence of occasional neurites was scored (-), small neurites with an area within 100x100 μm were scored (+) (Figure 4.5: D1-D2),neurites with an area greater than (+) but within 200x200 μm were scored as (++) (Figure 4.6: G1-G2 and H1-H2) and neurites occupying an area greater than 200x200μm were scored (+++). Only N2B27 medium following priming with the higher concentration of RA produced the highest score (Figure 4.6: J1-J2, Figure Figure 4.8 and Table 9).

In contrast with the results of the Embryoid Body-based differentiation method, these cultures showed a lower proportion of EAP3 positive than 3A10 positive cells (Figure 4.5, Figure 4.6, Figure 4.7, Figure 4.8 and Table 9). The greatest number of transitin-expressing cells was seen when N2B27 was used both in priming (with lower dose of RA) and differentiation. These findings show that N2B27 medium supplemented with high RA concentration can efficiently direct cES cells to neuronal differentiation in vitro.

Figure 4.5: Neural marker expression by cES cells cultured in N2B27 medium as an adherent monolayer (A)

A1-A4: Chick ES cells do not express neuronal (3A10) or glial (EAP3) markers when cultured for 3 weeks in standard cES medium [A2 & A4 respectively] (where they continue to proliferate). B1-E4: Chick ES cells start spontaneous differentiation on monolayer culture following withdrawal of cES cells prolifration maintaining BRL conditioned medium (BRL – ESA) wherebye a few numbers of cells expressing the neuronal marker 3A10 (but not the glial marker EAP3 (A4-E4)) appear after 20 days of culture in DMEM with low ($5x10^{-7}$ M) (C2) or high ($5x10^{-6}$ M) (D2) concentration of retinoic acid (RA). No cells expressing the neuronal or the glial markers were identified after 3 weeks of monolayer culture in DMEM alone (B2, B4 respectively). In contrast, growing the cells in the N2B27 medium for 18 days following an initial 2 days 'priming' period in DMEM yield many cells with neuronal morphology that express the post mitotic neuronal marker 3A10 (E2) (Scale bar = 100μm).

Figure 4.5: Neural marker expression by cES cells cultured in N2B27 medium as an adherent monolayer (A)

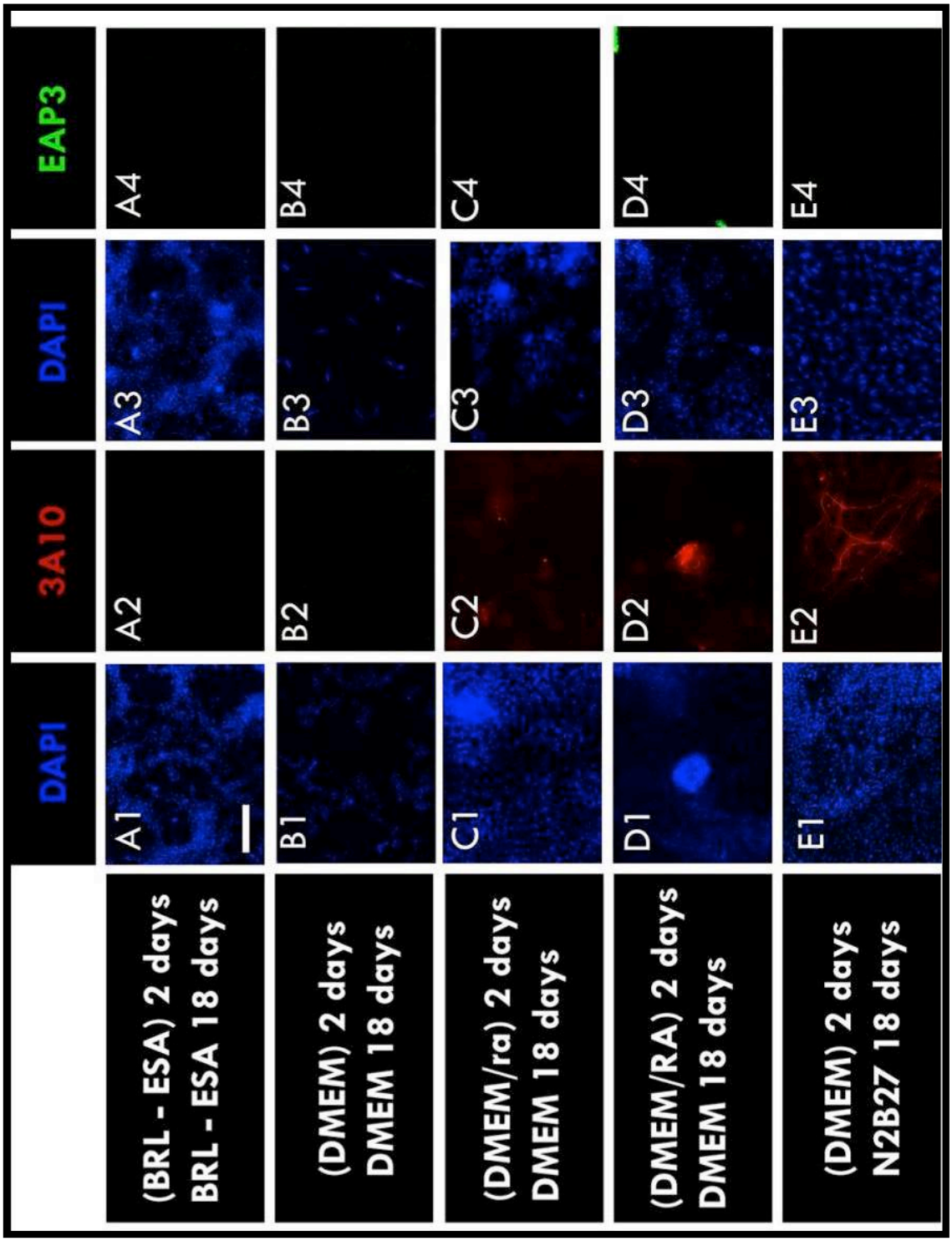


Figure 4.6: Neural marker expression by cES cells cultured in N2B27 medium as an adherent monolayer (B)

F1-G4: Chick ES differentiation into neurons in monolayer culture medium is more efficient when they are treated with RA during the 'priming' period. Cells expressing the neuronal marker 3A10 appear in more number when RA concentration used increases (Compare F2: low RA concentration ($5x10^{-7}$ M) to G2: higher RA concentration ($5x10^{-6}$ M). A more efficient differentiation of cES cells into cells expressing neuronal and/or glial markers is seen when the N2B27 medium was used in the 'priming' period at the beginning of the monolayer culture neuronal differentiation 21 days protocol (Compare I2 and J2 with F2 and G2). The highest level of differentiation for both markers was seen when cells were primed and cultured in the N2B27 defined medium with high concentration of RA used during the priming period (J2 and J4). (Scale bar = 100μm)

Figure 4.6: Neural marker expression by cES cells cultured in N2B27 medium as an adherent monolayer (B)

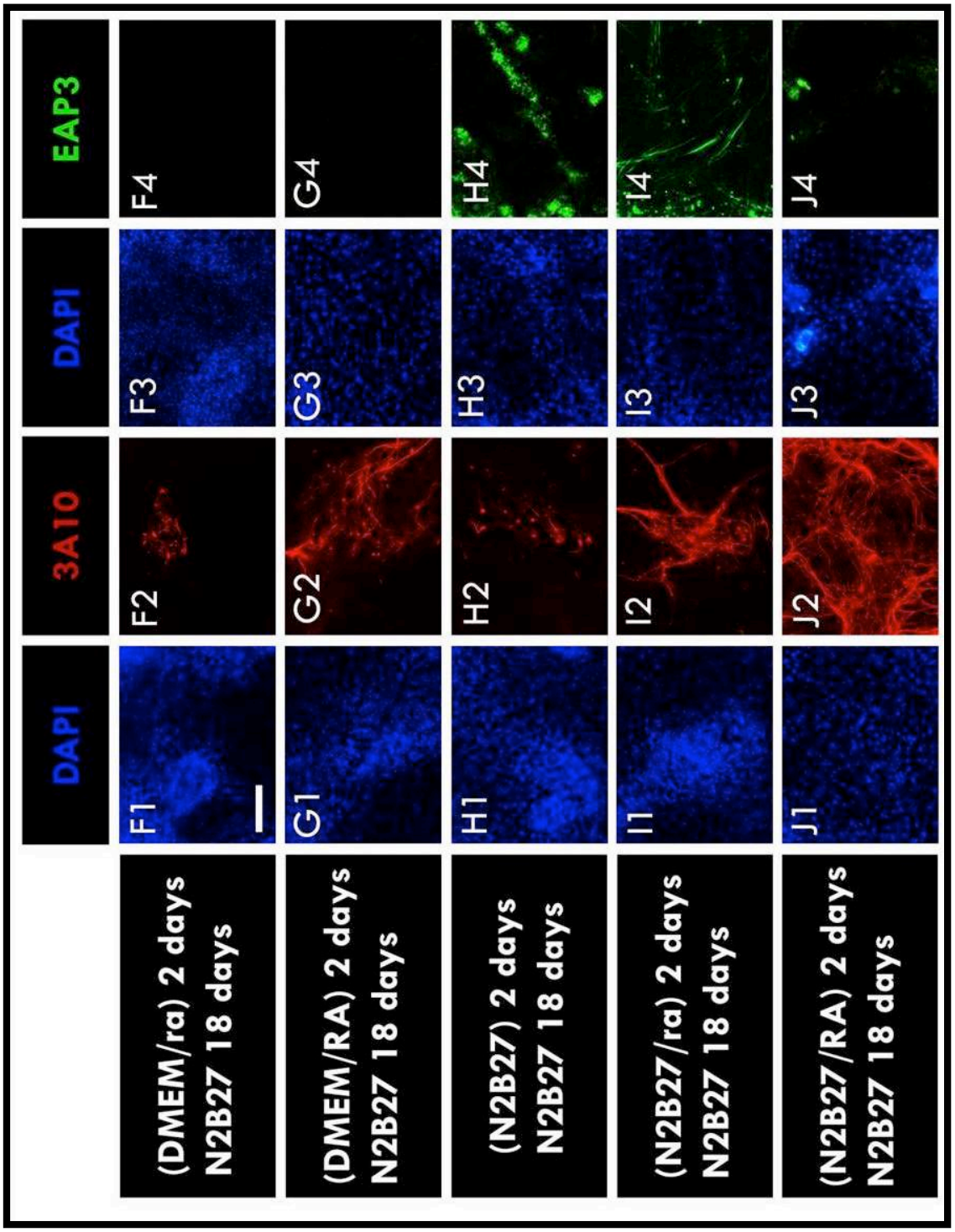


Figure 4.7: Various alterations of monolayer based protocol induced neural differentiation of cES cells.

Figure 4.7 summarizes the results of different protocols used to induce *in vitro* neuronal an or glial differentiation of cES cells. DMEM or N2B27 medium with or without RA were used in the 2 days priming period. This was followed by 18 days of cell culture as a monolayer in DMEM or N2B27 medium. The lowest level of differentiation for both markers was seen when cells were primed and cultured in plain DMEM medium. In contrast, the highest level of induced neuronal differentiation was seen when cells were primed and cultured in the N2B27 defined medium with high concentration of RA used during the priming period.

Figure 4.7: Various alterations of monolayer based protocol induced neural differentiation of cES cells.

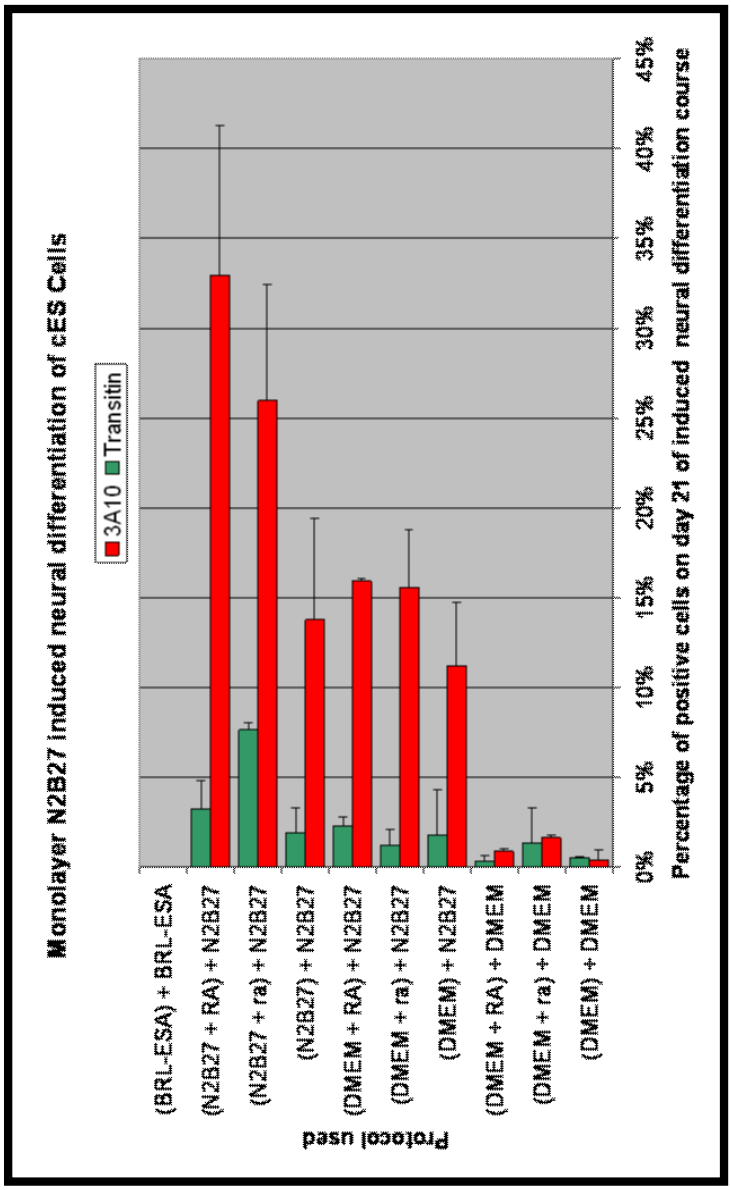
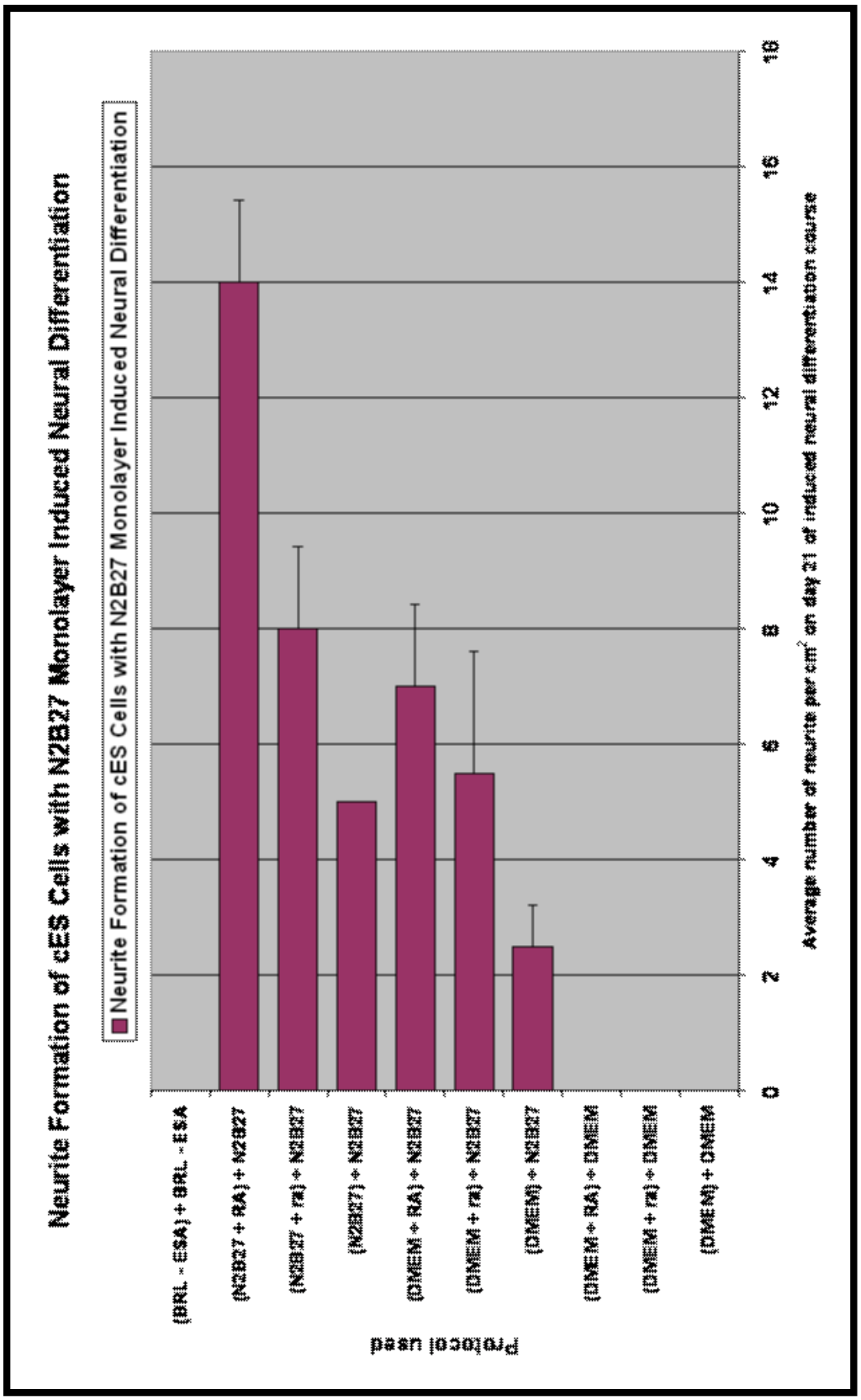


Table 9: Induced neuronal differentiation of cES cells in defined media .

Exp.I	Protocol sequence	RA	3A10+ cells (Mean%±SD)	EAP3+ cells (Mean%±SD)	Neurite formation (per cm^2) Mean ± SD	Neurite score
1	(DMEM) + DMEM		0.4 ± 0.6	0.5 ± 0.1	0.0 ± 0.0	-
2	(DMEM + ra) + DMEM	$5x10^{-7}M$	1.6 ± 0.1	1.4 ± 1.9	0.0 ± 0.0	-
3	(DMEM + RA) + DMEM	$5x10^{-6}M$	0.9 ± 0.1	0.3 ± 0.3	0.0 ± 0.0	-
4	(DMEM) + N2B27		11.2 ± 3.5	1.8 ± 2.5	2.5 ± 0.7	+
5	(DMEM + ra) + N2B27	$5x10^{-7}M$	15.6 ± 3.2	1.2 ± 0.9	5.5 ± 2.1	+
6	(DMEM + RA) + N2B27	$5x10^{-6}M$	15.9 ± 0.1	2.3 ± 0.5	7.0 ± 1.4	+
7	(N2B27) + N2B27		13.8 ± 5.7	1.9 ± 1.4	5.0 ± 0.0	++
8	(N2B27 + ra) + N2B27	$5x10^{-7}M$	26.0 ± 6.5	7.6 ± 0.4	8.8 ± 1.4	++
9	(N2B27 + RA) + N2B27	$5x10^{-6}M$	32.9 ± 8.3	3.3 ± 1.6	14.0 ± 1.4	+++
10	(BRL - ESA) + BRL - ESA		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	-

Figure 4.8: Neurite formation of cES cells following monolayer N2B27 medium induced differentiation protocol. Neurite formation was possible following *in vitro* induced neural differentiation of cES cells using a different combination of DMEM and N2B27 medium for priming and monolayer culture. The N2B27 defined medium used for priming cES cells (with higher concentration of retinoic acid) and subsequently in culturing them for a total of 21 days as a monolayer led to the most profuse neurite formation in comparison to other protocols.

Figure 4.8: Neurite formation of cES cells following monolayer N2B27 medium induced differentiation protocol.



4.3.3. Expression of molecular markers in cES cells

To assess the time course of neuronal differentiation, the expression profile of several markers was determined in cES cells during their in vitro neural differentiation using the [N2B27+RA] 2 days + N2B27 18 days protocol presented above. Cells were fixed and stained for neuronal markers (3A10 and TUJ-I) at day 0, 10 and 20 to compare the trend of neuronal marker expression. Neither 3A10 nor TUJ-I was expressed in proliferating cES cells, (Figure 4.9: A,B and I, J). After the first half of the time course both antigens are expressed in about 13% and 6% of cells respectively (Table 10, Figure 4.9 for 3A10: C,D,E and for TUJ-I: K,L,M and Figure 4.10). The proportion of expressing cells continues to increase to reach approximately 11% of differentiating cells in day 21 for TUJ-I and about a third of the cells for 3A10 (Figure 4.9 for 3A10 F, G, H and for 3A10 N, O, P as well as Figure 4.10 and Table 10). This time course suggests neural differentiation of cES cells starts soon after the withdrawal of conditions supporting proliferation.

Figure 4.9: Expression of neuronal molecular markers in N2B27 induced cES cells: Following in vitro induced neural differentiation using the [N2B27+RA] 2 days + N2B27 18 days protocol (See above). cES Cells does not stain for neuronal markers (3A10 and TUJ-I) during its proliferating 'stem' state (A,B and I, J). On the tenth day of the time course both antigens are expressed in about 13% and 6% of cells respectively (C, D, E (3A10) and K,L,M (TUJ-I)). The proportion of expressing cells continues to increase to reach approximately 11% of differentiating cells in day 21 for TUJ-I and about a third of the cells for 3A10 (F, G, H and N, O, P respectively). (Also see Figure 4.10 and Table 10) (Scale bar = 50μm).

Figure 4.9: Expression of neuronal molecular markers in N2B27 induced cES cells

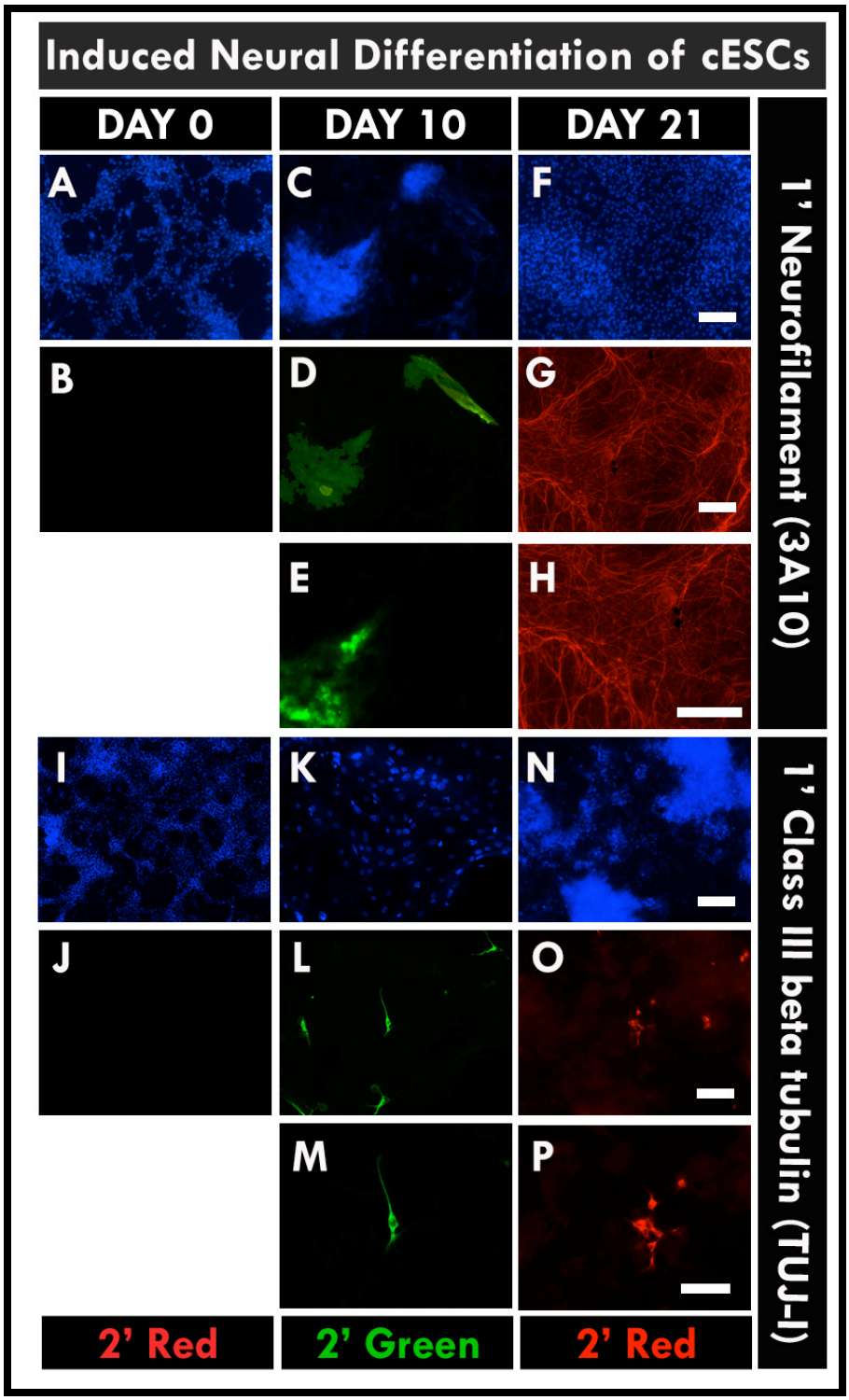


Figure 4.10: Expression of neuronal molecular markers in N2B27 induced cES cells (Quantification): Following in vitro induced neural differentiation using the [N2B27+RA] 2 days + N2B27 18 days protocol (See above). cES Cells does not stain for neuronal markers (3A10 and TUJ-I) during its proliferating ‘stem’ state . On the tenth day of the time course both antigens are expressed in about 13% and 6% of cells respectively. The proportion of expressing cells continues to increase to reach approximately 11% of differentiating cells in day 21 for TUJ-I and about a third of the cells for 3A10 (See also Table 10).

Figure 4.10: Expression of neuronal molecular markers in N2B27 induced cES cells (Quantification)

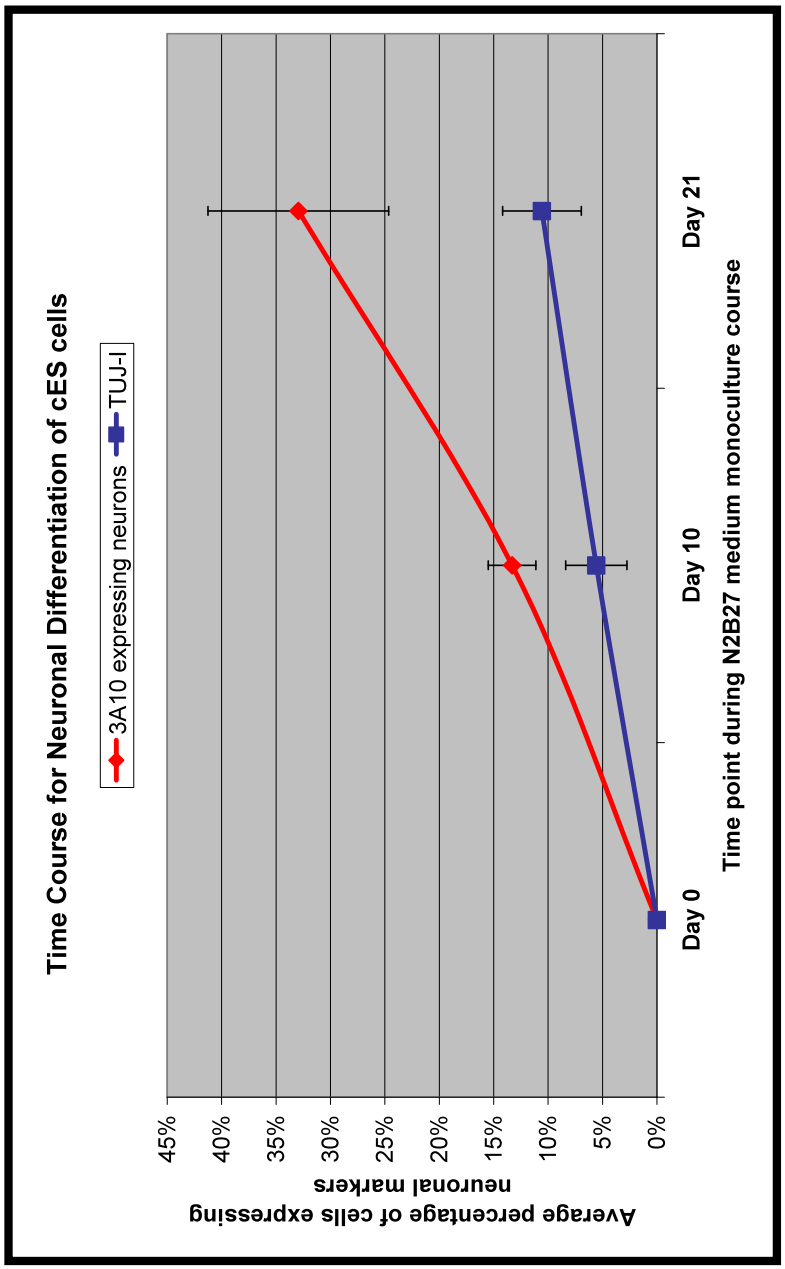


Table 10: Expression of neuronal (3A10 & TUJ-1) molecular markers in N2B27 induced cES cells.

			EXP 1			
Day/State	**3A10+ cells**	**Total Counted**	**%**	**TUJ-I**	**Total counted**	**%**
Day 0	0	1125	0.0%	0	882	0.0%
Day 10	72	612	11.8%	29	811	3.6%
Day 21	354	912	38.8%	55	419	13.1%
			EXP 2			
	3A10+ cells	**Total Counted**	**%**	**TUJ-I**	**Total counted**	**%**
Day 0	0	713	0.0%	0	1098	0.0%
Day 10	119	801	14.9%	48	635	7.6%
Day 21	143	528	27.1%	63	786	8.0%
			Total			
Day 0		0 ± 0 %			0 ± 0 %	
Day 10		13.3 ± 2.2%			5.6 ± 2.8 %	
Day 21		32.9 ± 8.3 %			10.6 ± 3.6%	

4.4. Discussion

The results presented in this chapter show that two methods for inducing mouse ES cells to differentiate into neural fates are also effective in cES cells, but to different extents. A method based on an intermediate step of Embryoid Body (EB) production is somewhat efficient. However a much greater proportion of cells differentiates into cells expressing neural markers using a monolayer culture method where cells are primed with a high concentration of Retinoic Acid for 2 days in the presence of the defined neuronal culture medium N2B27 (Brewer et al., 1993; Ying et al., 2003; Ying and Smith, 2003) followed by further culture in N2B27. Thus, chick embryonic stem cells are comparable to their mouse counterparts in that they can be directed to neural differentiation with different protocols. The ability to direct cES cells to neural differentiation in defined medium overcomes the objections to studying neural commitment and differentiation in EB or stromal cell based assays (Bain et al., 1995; Kawasaki et al., 2000). The monolayer/N2B27 method involves chemically defined media in the absence of serum, heterologous feeder cells or conditioned media.

Here, neural identity was assessed by expression of 3 markers: the neurofliament-associated marker 3A10 (Furley et al., 1990; Storey et al., 1992; Yamada et al., 1991), the neuronal precursor and glial cell marker EAP3 (McCabe et al., 1992; Napeir et al., 1999; Yuan et al., 1997) and the differentiating neuronal marker TUJ1 (Ferreira and Caceres, 1992;Maurer et al., 2007; Miura and Kameda, 2001; Scott et al., 1990) in a method based on monolayer cultures. Using 3A10 and TUJ1, the time course of acquisition of expression of these markers was assessed. Neuronal differentiation starts relatively early in the cultures, half way through the culture period. It is also worth noting that a smaller, but not insignificant amount of neural differentiation is seen in cultures kept in proliferation medium and/or when the priming and/or N2B27 steps are omitted. It will be important to determine

whether these conditions also support differentiation into other cell types to determine whether they particularly favour commitment into neural lineages.

N2B27 medium alone produced about 15% neuronal differentiation and this seemed to increase by using the higher concentration of RA. The mode of action of retinoic acid in promoting differentiation (or specifically neural differentiation) has not been well defined and is thought to involve multiple signalling pathways (Bally-Cuif et al., 1995; Endo et al., 2009; Lu et al., 2009;Ying et al., 2003)

The inducing properties of the N2B27 medium may also be due to the summation of multiple signalling pathways. The most obvious component is Insulin-like Growth Factor (IGF). This and its related factor Insulin activate the MAP kinase cascade which is shared by FGF and have been reported to have neural inducing activity in several species (Fischer et al., 2009; Freund et al., 2008; Pera et al., 2001). It is, therefore, conceivable that initial FGF (which is present in the proliferating-stimulating medium) followed by an FGF blocking signal (RA) and a low level of MAPK activation (IGF) is a cue for acquisition of neural fate [see also (Papanayotou et al., 2008; Sheng et al., 2003; Stern, 2005a; Stern, 2006)].

Chapter 5. Gene expression profile and activity of the N2 enhancer during induced neural differentiation of cES cells

5.1. Introduction

Chick ES cells are derived from apparently homogeneous epiblast cells of pre-streak embryos (Pain et al., 1996). Chick ES cells can differentiate into descendants of the three germ layers (ectorderm, mesoderm and endoderm) both *in vivo* (Van de Lavoir et al., 2006b) and *in vitro* (Pain et al., 1996). Although cells from stage X blastoderms (from which chick ES cells are derived) can generate both somatic and germ line chimaeras when transferred to recipient embryos (Petitte et al., 1990), their ability to contribute to the germ line is lost upon culture (Pain et al., 1996; Pain et al., 1999; Petitte et al., 2004; Van de Lavoir et al., 2006b).

Undifferentiated, proliferating chick ES cells can be characterised by their expression of markers such as telomerase and alkaline phosphatase activity as well as antigens such as SSEA 1 & 3 and ECMA-7 (Pain et al., 1996;Petitte et al., 2004) and transcription of genes including ERNI/ENS1 (Acloque et al., 2001), Oct4 and Nanog (Lavial et al., 2007). All of these markers are downregulated when cells are induced to differentiate (Acloque et al., 2001; Lavial et al., 2007). During the process of commitment to different cell types and the ensuing differentiation, the downregulation of these markers is accompanied by progressive acquisition of cell-type-specific markers. The differentiation of cES cells into mature cell types is a protracted process that takes several days (see Chapter 4), presumably reflecting that commitment to, and subsequent differentiation into, different cell types is a complex process comprising a hierarchy of distinct steps. A similar view has recently been

emerging from studies of the process of neural induction in the embryo (Papanayotou et al., 2008; Sheng et al., 2003; Stern, 2005b), although it is not yet clear whether neural induction in the embryo is an equivalent process to the acquisition of neural character by chick ES cells in vitro. One way to begin to address this question is to compare the time-course of changes in gene expression in the embryo and in ES cells that have been induced to differentiate. Here we study this during induced neural differentiation in chick ES cells (see also Chapter 4).

The regulation of the Sox2 gene is particularly interesting. It is expressed in pre-primitive streak chick and mouse embryos as well as in ES cells in the proliferative state, is then transiently downregulated before becoming expressed again in the neural plate of the embryo and in maturing neural precursors in vitro (Avilion et al., 2003; Bani-Yaghoub et al., 2006; Bertocchini et al., 2010a; Masui et al., 2007). In the embryo, Sox2 expression in the neural plate is initiated by activity of the N2 enhancer (Papanayotou et al., 2008; Uchikawa et al., 2003). Surprisingly, the same enhancer appears to be responsible for Sox2 expression in proliferating ES cells (Catena et al., 2004); see also (Chapter 3). It is therefore of particular interest to determine the time course of changes in activity of the N2 enhancer, as a prerequisite to identify the key transcriptional regulators that control its activity during the proliferative phase, commitment and subsequent differentiation. This Chapter addresses this question for the activity of N2, some of its regulators uncovered from studies on early embryos, and other markers of the ES cell state and of early neural commitment and differentiation.

In the embryo, with the help of fate maps one can study the gene expression profile hierarchy that precedes the development of a specific tissue/organ or a certain biological process by identifying the changes of gene expression of this tissue/organ from the earliest embryological ancestors following it through the stages of development until this tissue/organ and/or biological process has reached the developmental outcome specified.

To identify the gene expression hierarchy of a given set of genes associated with neural induction a similar approach can be employed following the epiblastic cells that are destined to become neural tissue in the adult. Table 11 is a compilation of data and published literature of the expression profile of the 10 markers studied in this section in medial epiblastic cells destined to become the anterior part of the adult central nervous system during stages X, XIII, 4+, 5+ and 6-9 of chick embryo development. State of expression of the specific genes are considered for future anterior CNS as per fate maps of forebrain development prepared by (Hatada and Stern, 1994)

In this chapter, we report the patterns of expression in cES cells of 10 genes involved in regulation of the stem state and linage commitment in chick embryo. Gene expression profiles are studied first under conditions that maintain their proliferative state. Secondly, we study and report the changes in mRNA expression of these genes are described during the 10 days following induction of neural differentiation in vitro. Finally, changes in the activity of the N2 enhancer of Sox2 are followed during this 10-day time course, and this compared with the expression of Sox2 mRNA.

5.2. Methods

5.2.1. Gene expression in cES cells

Ten genes were chosen that are normally expressed in the epiblast at the pre-primitive streak stage and whose expression changes during early stages of neural plate development. This set includes markers for endoderm (Sox17) and mesendoderm (Brachyury), as well as markers of ES cells (PouV [Oct3/4], Nanog, and ERNI), and markers of different stages of neural plate development: Sox2, Sox3, Sox1, Otx2 and one regulator of Sox2 expression in the developing neural plate (BERT; (Papanayotou et al., 2008). Expression was studied by in situ hybridisation as described in Chapter 2. Table 12 lists the probes, source and references.

In order to standardise the probes' signals plates of all days of the time course were prepared simultaneously. One the signal is developed and stopped in one well (corresponding to a specific day of the time course), then the revealing reaction of all wells corresponding to that gene in all other days would be stopped too. ISHn of whole embryos (at pre and post gastrulation stages) was used to calibrate the timing of signals. Staining reactions were stopped on the 6th day (See protocol in Chapter 2) if no signal developed.

For each experiment, six random (non-overlapping) 20x objective fields were scored. A 5-point scoring system was used to describe gene expression:

–: no expression detected expressed.
+: the marker is expressed in small cohorts of cells (<50% of the cells scored).
++: the marker is expressed more than half of the cells.
+++: expression in almost all of the cells
++++: as above, but also indicating particularly strong levels of expression.

For Sox2 mRNA expressing cells and N2 activity experiments, cells were counted as described in Chapter 2.

Table 11: Hierarchy of expression of ten genes involved in regulation of the stem state and linage commitment in the developing chick embryo.

	X	XIII	4+	5+	6+
ERNI	+	+	+	+	+
Oct3/4	+	+	+	+	+
Nanog	+	+	+	+	+
Sox1	-	-	-	-	+
Sox2	-	+	+	+	+
Sox3	+	+	+	+	+
Bert	-	-	+	+	+
Otx2	+	+	+	+	+
Bra	-	-	-	-	-
Sox17	-	-	-	-	-

During the first day of chick embryo development - from the stage of newly laid eggs [X: Eyal-Giladi & Kochav (1975)] up to 24 hours incubation [Stage 6+: Hamburger and Hamilton (1951; 1992)] – cells in the epiblast destined to form the anterior part of the central nervous system (forebrain) undergoes specific changes in their gene expression profile during early stages of neural differentiation. These changes are characterized by the upregulation neural markers (*Sox1, Sox2, Bert*) accompanied with upregulation of (*ERNI, Oct3/4, Nanog, Sox3* and *Otx2*) and continuous downregulation of genes upregulated in the other two germ layers at the same developmental stage (*Bra* in the mesoderm and *Sox17* in the endoderm). As a result, each cell of the embryo acquires a gene expressing profile that is specific to both its space and stage of development.

Table 12: Probes of gene markers used to study gene expression profile in differentiating cES cells

Insert name	Description	Cut emzyme	Transcribe enzyme	Source	Reference
ERNI Wpst	Subclose for ERNI for ISH	KpnI	T3	Cloned at C D Stern lab	Streit et al. 2000
cBra9	Chick Brachyury	XbaI	T3	Gift from J C Smith	Smith et al. 1991
BERT	Chick BERT	EcoRI	T3	Cloned at C D Stern lab	Papanayotou et al. 2008
Ip06	Chick Oct3/4	NcoI	SP6	Gift from B Bain	Lavial et al. 2007
PFL Nanong	Chick Nanog	ApaI	SP6	Gift from B Bain	Lavial et al. 2007
pBSXsox17α	Chick Sox17	SmaI	T7	Gift from Woodland	Hudson et al. 1997
cSox-3	Chick Sox3	PstI	T7	Gift of R. Lovell-Badge & P. Scotting	Uwanogho et al. 1995
cSox2	Chick Sox2	PstI, NcoI	T7	Gift of R. Lovell-Badge & P. Scotting	Uwanogho et al. 1995
cSox1	Chick Sox1	XhoI	T7	Gift of H Kondoh	Kamachi et al 1998
cOtx2	Chick Otx2	XhoI	T3	Gift of L. Bally-Cuif & E Boncinelli	Bally-Guif et al 1995

5.2.2. Induction of differentiation of cESCs

Chick ES cells were maintained in culture as described in Chapter 2. Neural differentiation was triggered by culture as a monolayer in N2B27 medium. However, cells cultured in N2B27+RA medium did not survive lipofectamine transfection (not shown). It turned out to be necessary to use N2B27 in the presence of 1% fetal calf serum.

5.2.3. Plasmid design

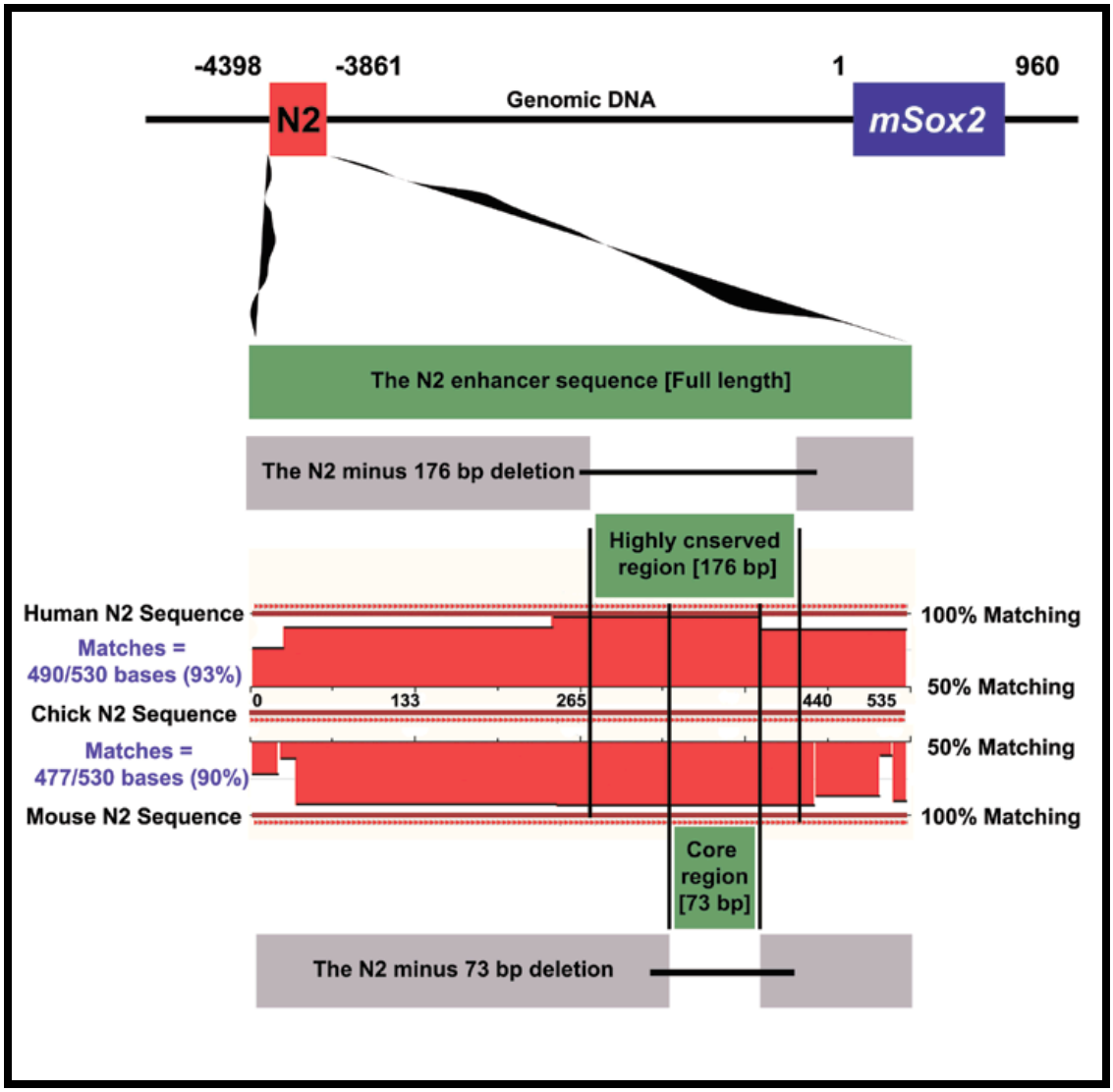
As described earlier in this thesis, pioneering work in Hisato Kondoh's laboratory led to the discovery of many distinct enhancers regulating different aspects of Sox2 expression (Uchikawa et al., 2003). Subsequent analysis of the N2 enhancer by the same group identified two essential sub-regions sufficient to drive the expression of a reporter in the area where Sox2 mRNA is normally expressed in the prospective neural plate just after gastrulation (Iwafuchi et al., 2008). These sub-regions are 176-bp and 73-bp long, the latter being contained within the former. They are highly conserved between chick, mouse and man.

To analyse the N2 enhancer and its two core sub-regions, as well as to align the corresponding sequences from different species, the Mulan software package (Loots and Ovcharenko, 2007) was used. The results of this analysis are presented in Figure 5.1 and Appendix 1.

Five reporters comprising: the full N2 region, the 176bp and 73bp core regions, and N2 lacking each of these regions, driving expression of EGFP were kindly provided by Professor Kondoh's laboratory. Figure 5.1. Line 9N2 of chick ES cells was transfected with Lipofectamine2000^{TM}. EGFP was visualised by fluorescence microscopy in unfixed, living cultures. Immunohistochemistry, ISH, cell counting and photography were done as described in Chapter 2.

Figure 5.1: Further dissection of the sequence of the N2 enhancer reveals 2 essential highly conserved core subregions: Subsequent analysis of the N2 enhancer by the same group identified two essential sub-regions sufficient to drive the expression of a reporter in the area where Sox2 mRNA is normally expressed in the prospective neural plate just after gastrulation. These sub-regions are 176-bp and 73-bp long, the latter being contained within the former. They are highly conserved between chick, mouse and man (See Appendix 1).

Figure 5.1: Further dissection of the sequence of the N2 enhancer reveals 2 essential highly conserved core subregions



5.2.4. Statistical analysis

A parametric ANOVA test was used to evaluate the activity of the reporter constructs during neural differentiation. A paired Student's t test was used for comparisons between pairs for plasmid activity. Pearson's r test was used to correlate between Sox2 mRNA expression and the activity of N2 during the time-course of induced neural differentiation. Figures are presented as mean ± SD and activity refers to the average percentage of green fluorescent cells in non-overlapping 20x objective fields in 2 independent experiments, performed on different days using different batches of cells (See Chapter 2).

5.3. Results

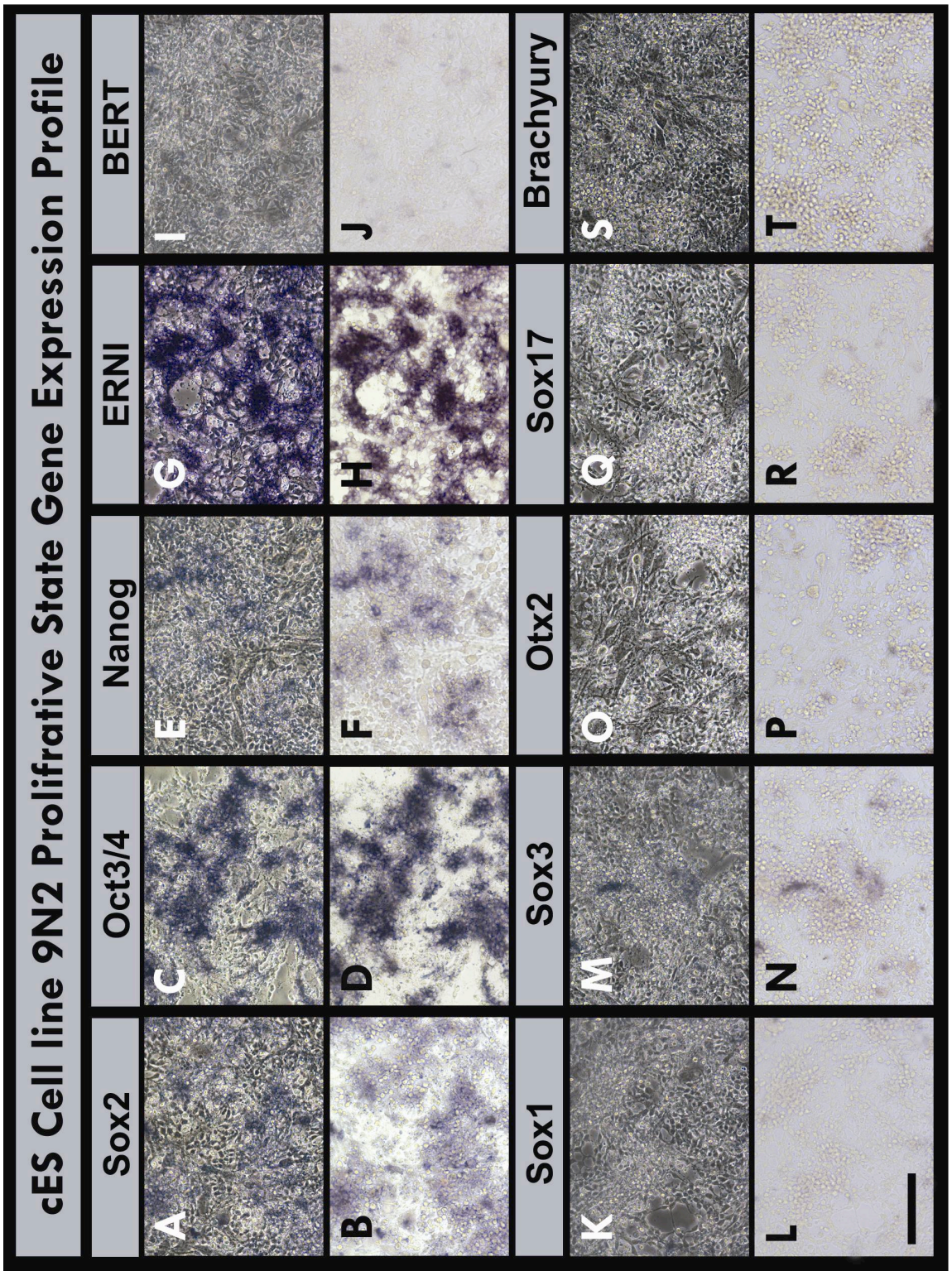
5.3.1. Patterns of gene expression during induced neural differentiation

5.3.1.1. Gene expression profile of proliferating 9N2 cES cells

Proliferating 9N2 cells express *Sox2*, *PouV [Oct3/4]* and *Nanog* (Figure 5.2 A-F). Most cells express of Sox2 mRNA [scored as +++] but the intensity of staining is variable (Figure 5.2 A, B). Expression of *PouV* mRNA is also generalised and strong [+++] (Figure 5.2 C, D). *Nanog* expression is more variable [+/++] and the intensity of staining varies between groups of cells within the same culture (Figure 5.2 E, F). *ERNI* is very strongly expressed in almost all cells [++++] (Figure 5.2 G, H). Sox3 mRNA is expressed by a sub-set of cES cells [+/++] (Figure 5.2 M, N). Otx2 is expressed in a small subset of cells [+] (Figure 5.2 O, P). Proliferating cES cells do not express *BERT* (Figure 5.2, I, J), *Sox1* (Figure 5.2 K, L), *Sox17* (Figure 5.2 Q, R) or *Brachyury* (Figure 5.2 S, T) [- in all cases] (Scale bar = 100μm).

Figure 5.2: Gene expression profile of the 9N2 cES cell line in the proliferative 'stem' state: Proliferating 9N2 cells express *Sox2*, *PouV [Oct3/4]* and *Nanog* (A-F). Most cells express of Sox2 mRNA [scored as +++] but the intensity of staining is variable (A, B). Expression of *PouV* mRNA is also generalised and strong [+++] (C, D). *Nanog* expression is more variable [+/++] and the intensity of staining varies between groups of cells within the same culture (E, F). *ERNI* is very strongly expressed in almost all cells [++++] (G, H). Sox3 mRNA is expressed by a sub-set of cES cells [+/++] (M, N). Otx2 is expressed in a small subset of cells [+] (O, P). Proliferating cES cells do not express *BERT* (I, J), *Sox1* (K, L), *Sox17* (Q, R) or *Brachyury* (S, T) [- in all cases].

Figure 5.2: Gene expression profile of the 9N2 cES cell line in the proliferative 'stem' state



5.3.1.2. Gene expression during induced neural differentiation

For these experiments, neural differentiation of cES cells was induced by culture in N2B27 medium without retinoic acid in the presence of 1% fetal calf serum (see above). Changes of gene expression were followed over a 10-day time course following induced neural differentiation. The results are presented in Table 13, Figure 5.3, Figure 5.4, Figure 5.5 and Figure 5.6.

Following application of N2B27 medium, cES cells downregulate expression of ES cell markers *Sox2*, *PouV*, *Nanog* and *ERNI* (Figure 5.3, Figure 5.4). In the first 3 days following induced neural differentiation, *Sox2* expression weakens (Table 13, Figure 5.3: A-F, M-P). By day 4 *Sox2* is weakly expressed in the centre of islands of particularly confluent cells (Table 13, Figure 5.3: Q, R). Between days 5-7 expression starts to involve more cells, peaking at day 7 (Table 13, Figure 5.3: G-J, S-T). From day 8 to day 10 *Sox2* is progressively downregulated (Table 13, Figure 5.3: K, L, U, V). By day 10 there is a marked change of cell morphology, cultures now containing many needle-like cells (Figure 5.3: K, W) and *Sox2* expression being restricted to small clumps of cells (Table 13, Figure 5.3: K, L, W, X).

The expression of *PouV*, *Nanog* and *ERNI* also decreases in the first half of the time-course (Table 13, Figure 5.4: G-J, M-P, S-V). In the second half of the time course expression of these markers continues to decrease (unlike the upregulation seen for Sox2 from day 4-5) (Table 13, Figure 5.3, Figure 5.4: K, L, Q, R, W, X).

The expression of pre-neural and early neural plate markers (*Otx2*, *Sox3, Sox1* and *BERT*) also undergoes dynamic changes. *Otx2* and *Sox3* did not change during the time course: clumps of cells expressing these markers were found at all time points (Table 13, Figure 5.5: G-L, M-R). Although the cell clumps expressing these two markers looked more prominent in some fields towards the very end of the time course (Figure 5.5: K, L, Q, R) these increases were not uniform and did not affect the score

BERT and *Sox1* were both upregulated during the time course (Table 5.2, Figure 5.5: A-F, S-X). *Sox1* expression first appeared at day 5 (Table 13, Figure 5.5: A-D) and remained, albeit weakly, until the end of the time course (Table 13, Figure 5.5: E, F). *BERT* started to be expressed between days 4-5 (Table 13, Figure 5.5: S-V). However, in contrast with the persistently weak expression of *Sox1*, *BERT* expression becomes stronger over days 6-7 before weakening towards the end of the time course (Table 13, Figure 5.6: I-P).

Brachyury expression first appears weakly on day 5 (Table 13, Figure 5.6: A-D). This expression quickly increases over the next 2 days and becomes expressed in almost half the cells by day 7 (Table 13, Figure 5.6: E, F). Expression then remains high until the end of the time course (Table 13, Figure 5.6: E, F). In contrast no expression of the endodermal marker *Sox17* was observed during the entire time course (Table 13, Figure 5.6: Q-V).

Table 13: Expression profiles of 10 marker genes during induced neural differentiation of cES cells

	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
ERNI	++++	+++	+++	++	++	++	+	+	+	+	+
Oct3/4	+++	++	++	+	+	+	+	+	+	+	+
Nanog	+++	++	++	++	+	+	+	-	-	-	-
Sox1	-	-	-	-	-	+	+	+	+	+/-	+/-
Sox2	+++	-	-	-/+	+	++	+++	+++	++	+	-
Sox3	+/++	+	+	+	+	+	+	+	+	+	+
Bert	-	-	-	-	+	+	++	++	+	+/-	+/-
Otx2	+	+	+	+	+	+	+	+	+	+	+
Bra	-	-	-	-	-	-/+	+	++	++	++	++
Sox17	-	-	-	-	-	-	-	-	-	-	-

Gene expression profile of cES cells changes during the first 10 days of a 21-day *in vitro* induced neural differentiation protocol by monolayer culture in the N2B27 medium. During this period, cES cells undergo downregulations of the genes associated with the pluripotent proliferative state (*ERNI, Oct3/4* and *Nanog*). *Sox2* has a bi-phasic expression profile with early downregulation in the first three days followed by upregulation (day 3-7) then a late downregulation (day 8-10). The late neural marker *Sox1* and *Bert* are upregulated in the second half of the time course. The mesodermal marker *Bra* is also upregulating in the second half of the time course suggesting that the N2B27 medium does not bias cells to induced neural differentiation exclusively. *Sox3* and *Otx2* are upregulated without detected changes of expression during the time course and *Sox17*, which is expressed in the endoderm during early stages of chick embryo development, continues to be downregulated in cES cells during the 10-days time course (Compare with table 5.1 for expression of these genes in ectodermal cells destined to form the forebrain during early stages of chick embryo development)

Figure 5.3: Sox2 expression during induced neural differentiation of cES cells.

By in-situ hybridization (ISH) - [Each time point is represented by two pictures, the top e.g. Day 0 – A is taken with phase contrast microscopy setting, and the bottom e.g. Day 0 – B is taken with bright field microscopy setting for better elaboration of the ISH signal color], *Sox2* mRNA has a bi-phasic expression profile with early downregulation in the first three days followed by upregulation (day 3-7) then a late downregulation (day 8-10). The number of cells expressing *Sox2* mRNA in cESCs during induced neural differentiation was noted to change with initial decrease in the first three days of a 10-days time course (A-F, M-P); then an increase on the fourth day of the time course (Q-R) peaking on day 7 (I, J); then another decrease in the last third of the time course (Days 8 – 10: K-L, U-X) (Scale bar = 80μm).

Figure 5.3: Sox2 expression during induced neural differentiation of cES cells.

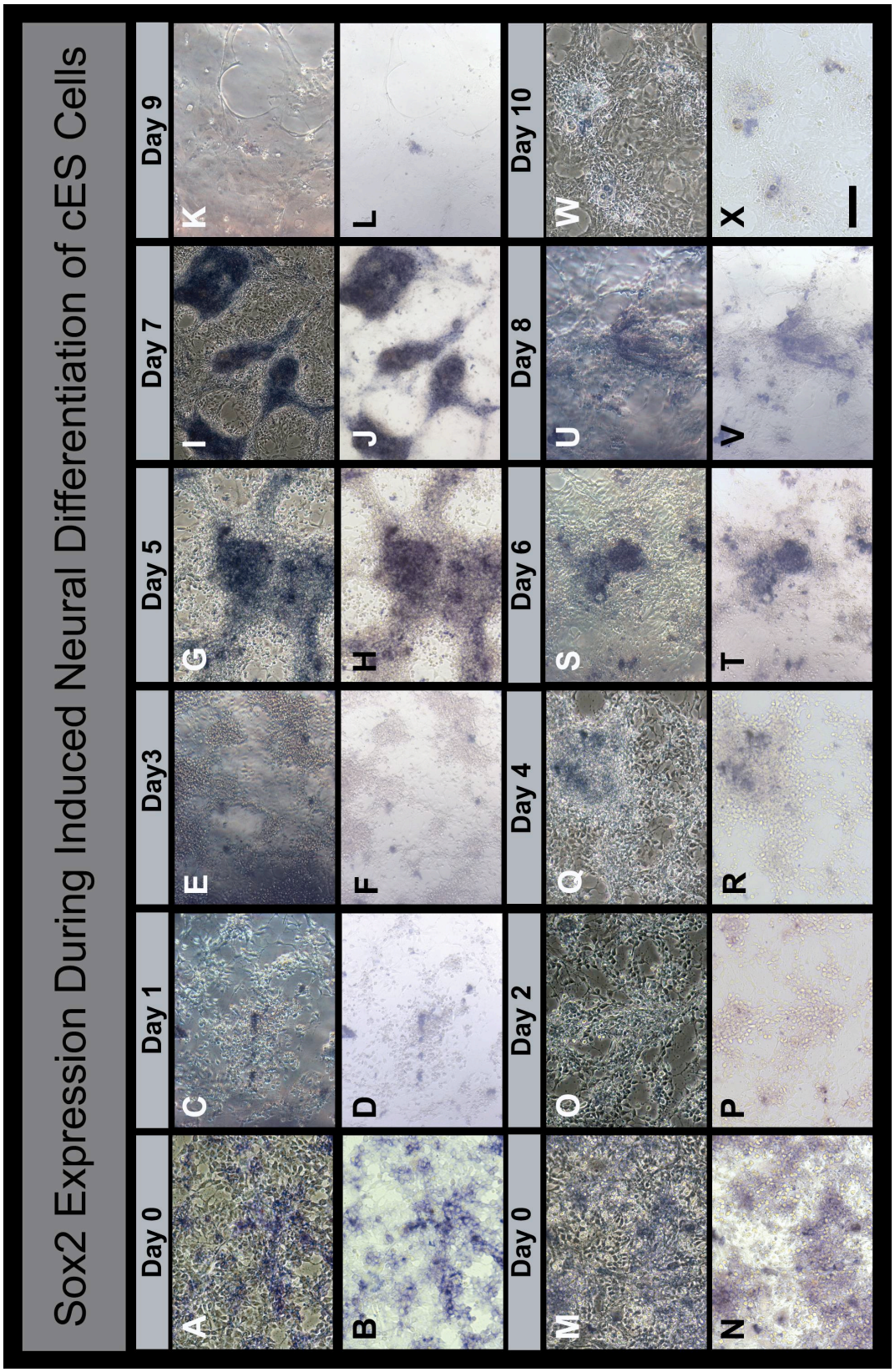


Figure 5.4: Changes of gene expressions profiles of cES cells 'stem' state markers during induced neural differentiation

ISH of cES cells mRNA gene expression during the first 10 days of cES cells induced neural differentiation demonstrated here with the blue colour of the ISH mRNA probes at the start (base proliferative stage - Day 0), mid-point (Day 5) and last day (Day 5). In contrast with the bi-phasic expression profile of *Sox2* mRNA, which is upregulated in the proliferative stage (A, B); at the midpoint (C, D) and downregulated on the last day (E, F), the mRNA expression of other proliferative 'stem' state marker genes *Oct3/4, Nanog, ERNI* is progressively downregulated during this time course (G-L, M-R and S-X respectively) (Scale bar = 100μm).

Figure 5.4: Changes of gene expressions profiles of cES cells 'stem' state markers during induced neural differentiation

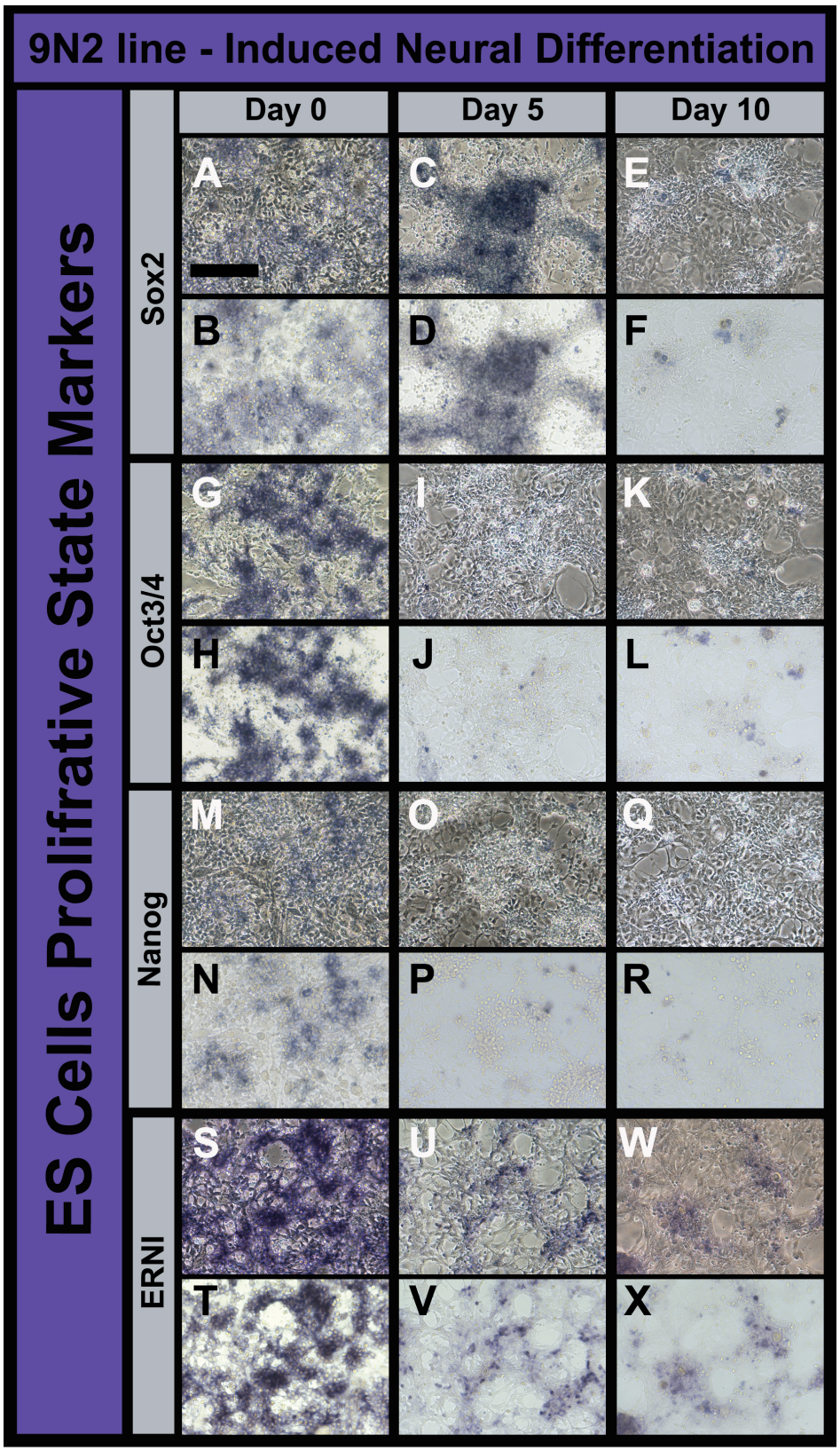


Figure 5.5: Changes of gene expressions profiles of cES cells 'pre-neural' & 'neural' state markers during induced neural differentiation.

ISH of cES cells mRNA of ‘pre-neural’ and ‘neural’ gene expression during the first 10 days of cES cells induced neural differentiation show upregulation of *Sox1* (A-F: compare the bright filed images on Day 0 (B) with that of day 5 (D)). A number of cES cells express the ‘pre-neural’ state markers *Sox3* and *Otx2* throughout the time course with no apparent changes at different time points (G-L and M-R respectively). *Bert*, the coiled-coiled domain partner of *ERNI* that is implied in *Sox2* mRNA regulation during neural induction of the chick embryo, is downregulated in the proliferative ‘stem’ state of cES cells (S, T) and is upregulated during the first half of the 10-day course (U-V) (Scale bar = 100μm).

Figure 5.5: Changes of gene expressions profiles of cES cells 'pre-neural' & 'neural' state markers during induced neural differentiation.

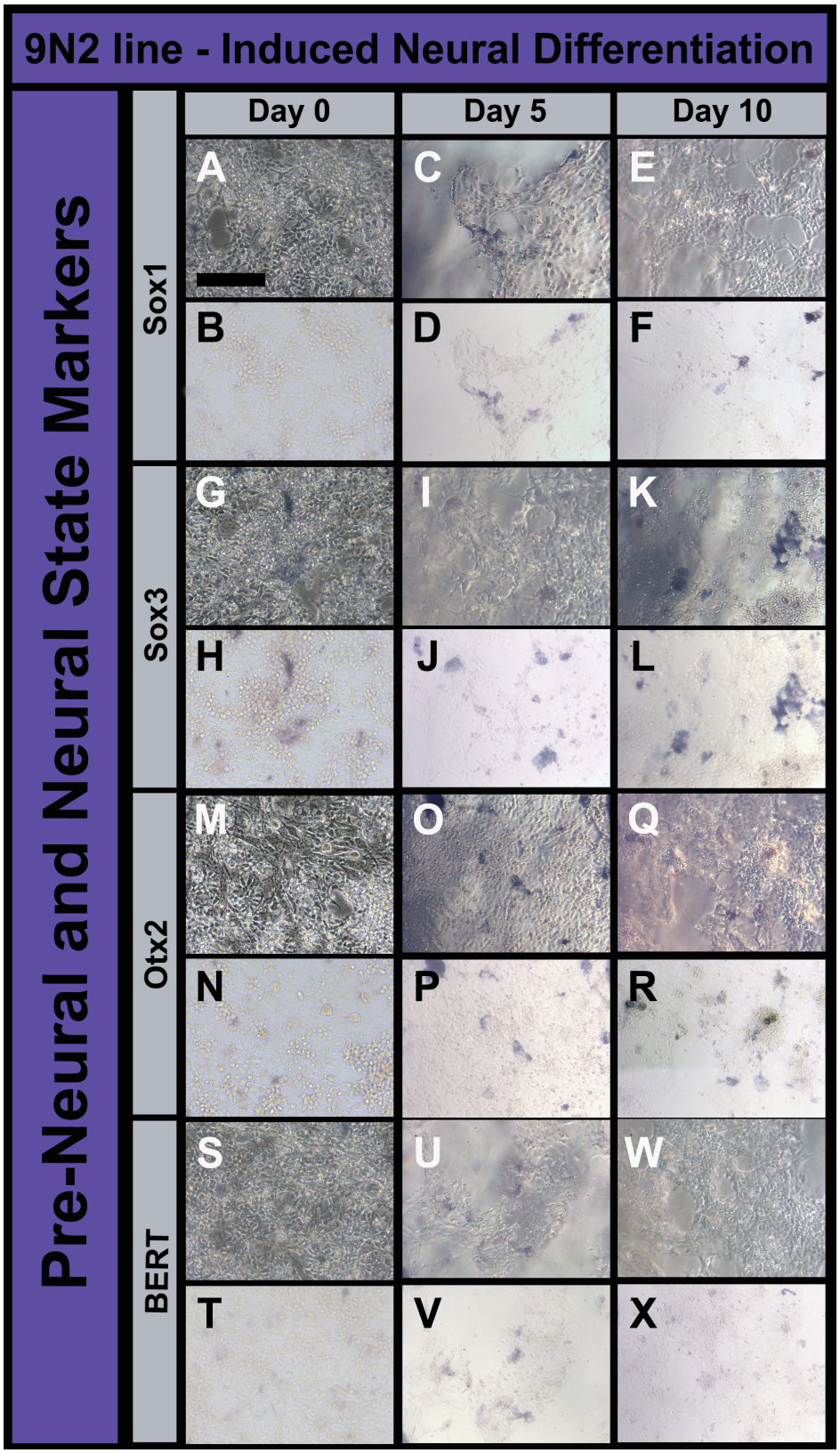
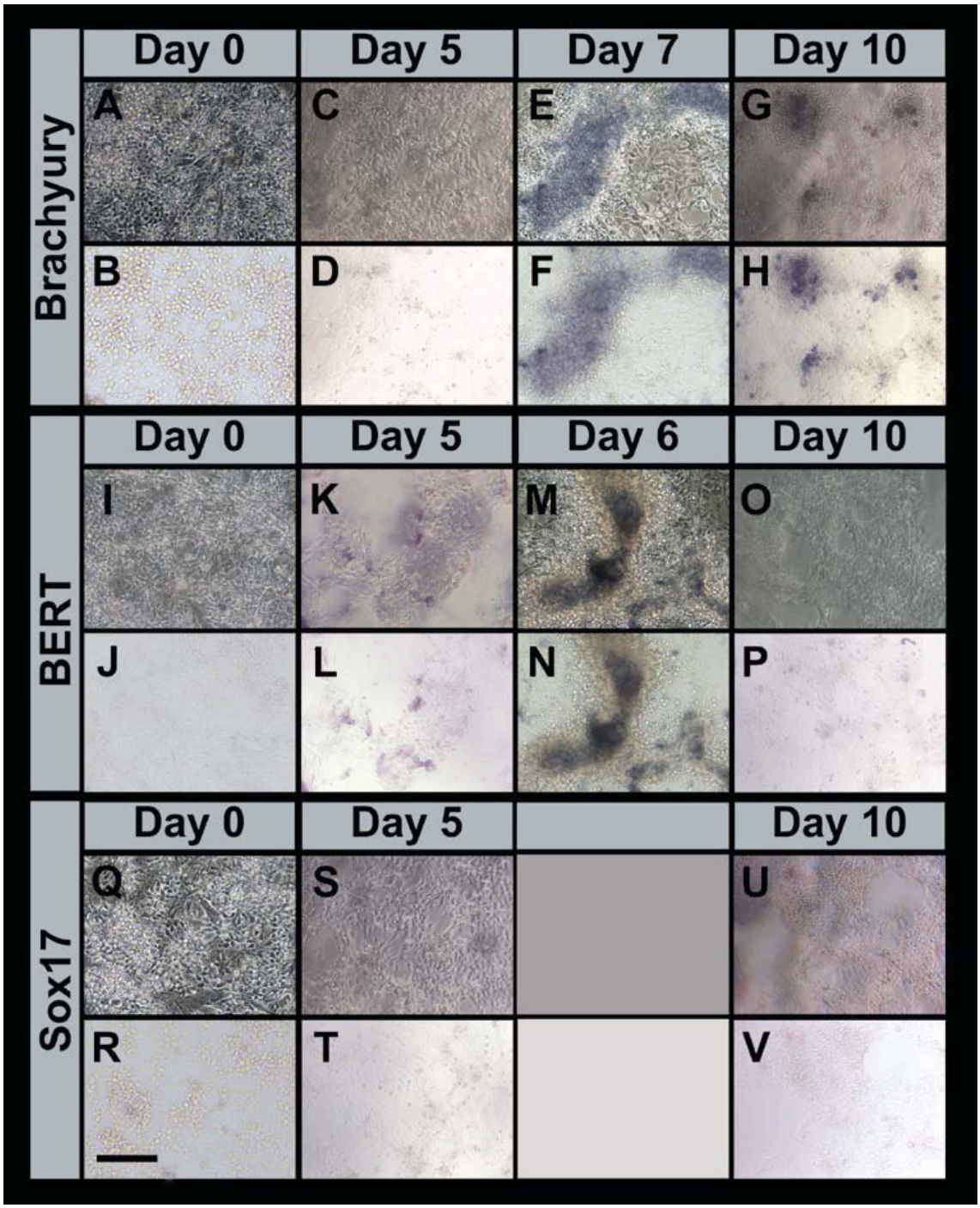


Figure 5.6: Expression of mesodermal (cBra), endodermal (cSox17) and ectodermal (Bert) markers in cES cells during induced neural differentiation.

ISH of cES cells during the first 10 days of cES cells induced neural differentiation. The mRNA of 3 genes expressed in the embryonic three germ layers during early neural induction of the chick embryo is shown. *Brachyury* (*Bra*), which is a mesodermal marker, is not expressed in cES cells during the proliferative 'stem' state (A, B) or in the first half of the time course (C, D). The N2B27 medium does not lead to exclusive induction of neural tissue as *Brachyury* is upregulated on day 7 (E, F) and continues to be so until the end of the time course (G, H). *Bert*, the coiled-coiled domain partner of *ERNI* that is implied in *Sox2* mRNA regulation during neural induction of the chick embryo, is expressed exclusively in the embryonic ectoderm during early stages of neural induction in the embryo (Papanayotou et. al., 2008). In proliferating cES cells *Bert* is not expressed (I, J). When cES cells are induced to differentiate into neurons, *Bert* is upregulated during the second half of the time course (I-P). In their proliferative 'stem' state, cES cells do not express the endodermal transcription factor *Sox17,* (Q, R). This continues to be the case throughout the 10 days time course (S, T & U, V) (Scale bar = 100μm).

Figure 5.6: Expression of mesodermal (cBra), endodermal (cSox17) and ectodermal (Bert) markers in cES cells during induced neural differentiation.



5.3.2. Activity of the N2 enhancer and its core subregions during induced neural differentiation

The profile of changes in the activity of different plasmids used in this experiment during a 10 day time course induced neural differentiation of 9N2 cells is presented in Table 14, Table 15, Table 16, Figure 5.7, Figure 5.9, Figure 5.10 and Figure 5.11.

5.3.2.1. Assessing transfection efficiency

To assess the efficiency of the transfection procedure, cells were transfected with pCAβ, a vector in which GFP expression is driven by a ubiquitously expressed promoter (chicken β-actin promoter) intensified by a CMV enhancer. The mean transfection efficiency for all experiments was 50.1 ± 11.4% (not shown in Table 14). In the proliferative state, average transfection efficiency was 50.1 ± 10.4 (Table 14, Figure 5.7: A-C). During the time course of induced neural differentiation, transfection efficiency varied between days with a range of 43.4 – 62.3% (Table 15; Figure 5.9: A1-10, B1-10; Figure 5.10 and Figure 5.11). By analysis of variance (ANOVA), the variation seen in transfection efficiency between days was not statistically significant (Table 15, Figure 5.9, Figure 5.10 and Figure 5.11).

5.3.2.2. Chick N2-tk-EGFP

The cN2 enhancer was expressed in 15.5 ± 0.7% of proliferating cES cells (Table 14 and Figure 5.7: D-F). After changing the culture conditions to induce neural differentiation, cN2 expression changed within the range 0.8–28.9% (Table 15 and Figure 5.11: B). The number of cells expressing started to increase after day 3, peaked on day 6 then decreased until the end of the time course (Figure 5.11: B). These changes were statistically significant (Table 15).

5.3.2.3. Mouse N2-tk-EGFP

The mN2 enhancer was expressed in 13.1 ± 1.2% of proliferating cES cells (Table 14 and Figure 5.7: G-I). Similar changes were seen after changing the culture conditions to induce neural differentiation as with the chick N2 enhancer construct (Table 15; Figure 5.9: C1-10; D1-10 and Figure 5.11: C). The number of cells expressing mN2 started to increase after day 3 (Figure 5.9: C4, D4, Figure 5.10 and Figure 5.11: C), peaked on day 6 (Figure 5.9: C6, D6; Figure 5.10 and Figure 5.11: C) then decreased until the end of the time course (Figure 5.9: C7-10 & D7-10; Figure 5.10 and Figure 5.11: C – compare with B). These changes were statistically significant (Table 15: Analysis of variance in the activity of the N2 enhancer and subregions in cES cells) but there was no significant difference between cN2 and mN2 on days 0, 5 and 10 (Table 16; Figure 5.9 and Figure 5.10).

5.3.2.4. Mouse N2[176bp]-tk-EGFP

The mouse N2 [176bp] sub-region was expressed by 8.7 ± 4.3% cES cells in the proliferating state (Table 14 and Figure 5.7: J-L). The proportion of expressing cells varied during the time course from 0.5 – 26.6% (Table 15; Figure 5.9: E1-10 & F1-10; Figure 5.10 and Figure 4.2: D). Similar changes were seen to those described for the full N2 (Figure 5.9; Figure 5.10 and Figure 5.11). Again the changes were statistically significant (Table 15). No significant difference was seen between the proportion of cells expressing mN2[176bp] and to mN2 [Full length] on days 0, 5 and 10 (Table 16; Figure 5.9 and Figure 5.10).

5.3.2.5. Mouse N2[73bp]-tk-EGFP

The results were similar to those with the longer core sub-region, with 7.7 ± 4.2% cells expressing this reporter (Table 14 and Figure 5.7: M-O). Upon neural differentiation a range of 1–21.1% cells showed activity (Table 15; Figure 5.9: G1-10 & H1-10; Figure 5.10 and Figure 5.11: E), and this changed

with a similar time-course to the other reporters described above (Figure 5.9; Figure 5.10 and Figure 5.11). There was no significant difference between the activities of the two sub- regions of N2 (Table 16 and Figure 5.10).

5.3.2.6. Mouse [N2 DEL-176bp]-tk-EGFP

A construct containing the N2 region from which the longer (176bp) sub-region had been deleted led to few cells expressing the reporter: 0.8 ± 0.2% (Table 14 and Figure 5.7: Q-S). This did not change during the time course (Table 15; Figure 5.9 and Figure 5.10).

5.3.2.7. Mouse [N2 DEL-73bp]-tk-EGFP

Deletion of the smaller sub-region from the mN2 reporter similarly led to few expressing cells (0.9 ± 1%; Table 14 and Figure 5.7: T-V) and no significant changes during the time course of differentiation (Table 15; Figure 5.9; Figure 5.10 and Figure 5.11).

Figure 5.7: Activity of the N2 enhancer and its subregions in proliferating cES cells

A-C: Following transfection of the cES cells with the various tk-EGFP plasmids the reporter gene Green fluorescence Protein (GFP) was detected using Immunohistochemistry (Positive control pCAB-GFP). Transfection with the full-length chick sequence of the N2 enhancer coupled to tk-EGFP reporter system (Chick-N2-tk-EGFP) resulted in the largest number of GFP positive cells (E, F). The use of the mouse sequence of the N2 enhancer in the same system (Mouse-N2-tk-EGFP) yielded similar results confirming conserved functional elements between the two species. In experiments fragments containing different deletions of the N2 mouse enhancer sequence was used GFP was detected only when a [176 bp] region (K, L) or a smaller [73 bp] core region (N, O), which is contained in former region, were used with the tk-EGFP reporter system. Minimal reporter gene protein was detected when these two regions were deleted from the N2 full length sequence used in the tk-EGFP system [N2 – 176 bp] (Q, R) and [N2 – 73 bp] (T, U) (Scale bar = 80μm)

Figure 5.7: Activity of the N2 enhancer and its subregions in proliferating cES cells

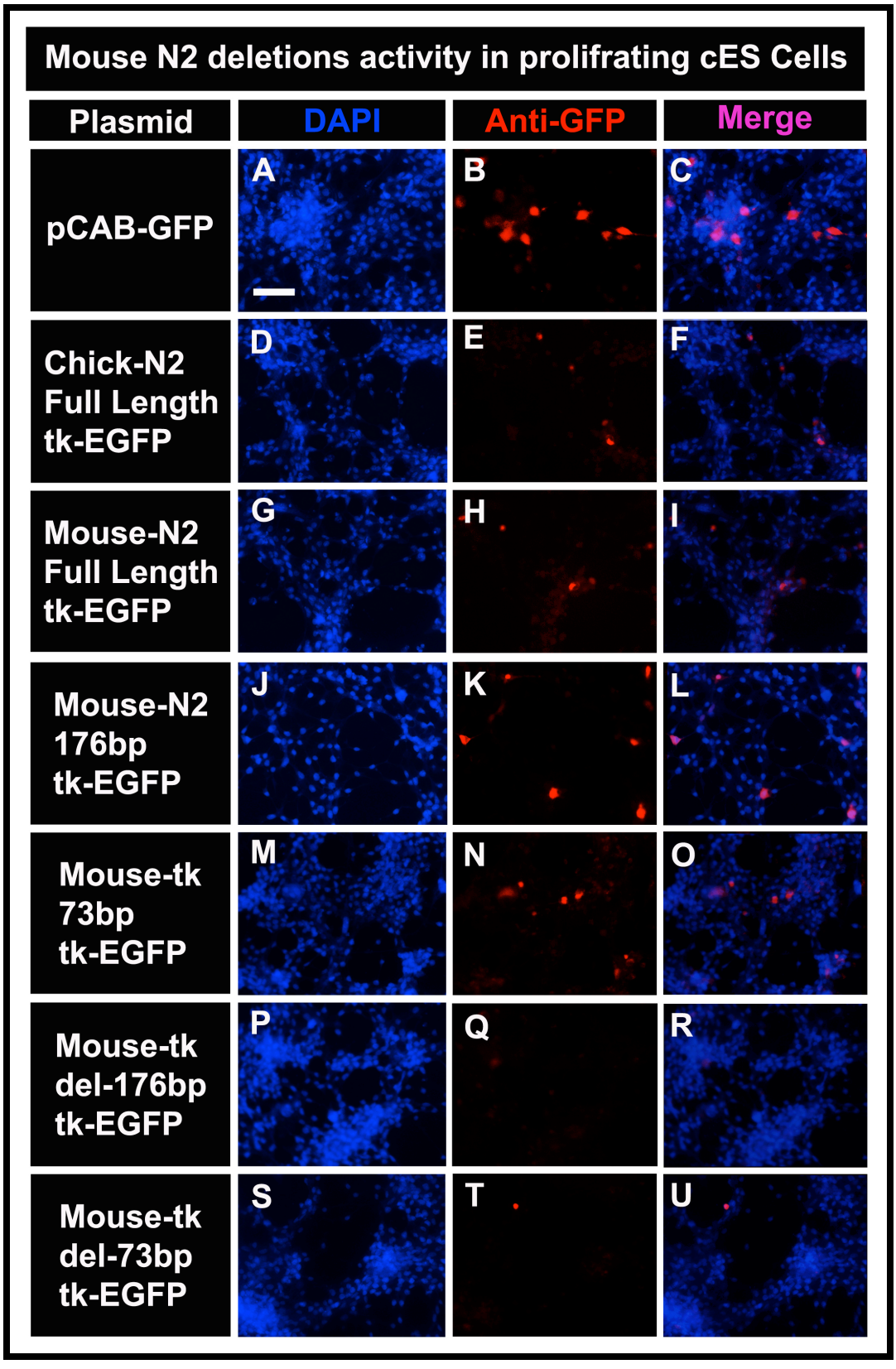


Table 14: Analysis of the activity of the N2 enhancer and its core subregions during induced neural differentiation

Plasmid	Experiment	Outcome	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
pCAB-GFP	Average	Mean percentage of GFP + cells	50.1	43.4	53.6	61.8	49.2	56.3	53.4	51.4	53.1	55.5	62.3
		SD of percentage of GFP + cells	10.4	5.7	12.2	1.3	1.2	7.2	8.0	5.1	6.8	2.9	6.3
	Experiment1	Positive cells counted 3x fields	557	335	1727	1353	943	857	1063	1058	587	829	1180
		All cells counted 3x fields	938	846	2793	2163	1930	1551	1849	2034	1171	1358	1667
	Experiment 2	Positive cells counted 3x fields	571	521	796	787	1029	489	590	706	612	1320	550
		All cells counted 3x fields	1326	1363	1647	1266	2124	957	1233	1538	1192	2406	868
cN2-tk-EGFP	Average	Mean percentage of GFP + cells	15.5	14.5	13.4	13.2	21.9	25.4	28.9	20.0	15.4	9.1	0.8
		SD of percentage of GFP + cells	0.7	4.6	5.9	3.6	1.6	3.4	7.2	0.5	6.9	2.1	0.8
	Experiment1	Positive cells counted 3x fields	175	97	99	239	248	278	330	214	137	111	16
		All cells counted 3x fields	1166	897	1074	1553	1235	1000	1345	1064	1319	1665	1269
	Experiment 2	Positive cells counted 3x fields	175	186	286	142	374	296	306	275	294	132	4
		All cells counted 3x fields	1062	1076	1749	1342	1515	1377	907	1426	1455	1348	1555
mN2-tk-EGFP	Average	Mean percentage of GFP + cells	13.1	12.4	10.8	11.2	23.1	23.2	25.1	21.9	10.3	3.9	0.4
		SD of percentage of GFP + cells	1.2	5.5	0.2	2.8	8.8	4.5	4.1	5.1	0.3	0.1	0.1
	Experiment1	Positive cells counted 3x fields	119	146	158	142	225	310	313	272	105	24	4
		All cells counted 3x fields	1281	1599	1517	1553	1342	1177	1287	1466	1035	891	723
	Experiment 2	Positive cells counted 3x fields	120	261	176	179	376	323	405	454	93	29	2
		All cells counted 3x fields	977	1458	1856	1342	1343	1672	1390	1621	1122	856	752
mN2[176bp]-tk-EGFP	Average	Mean percentage of GFP + cells	8.7	10.7	12.4	14.9	24.2	19.4	26.6	11.8	10.5	1.4	0.5
		SD of percentage of GFP + cells	4.3	8.6	9.4	3.0	1.9	2.9	3.5	6.8	3.9	0.4	0.2
	Experiment1	Positive cells counted 3x fields	204	162	209	157	291	278	437	125	123	30	5.0167
		All cells counted 3x fields	1837	1541	1825	1406	1089	1583	1632	1912	1277	2684	1231
	Experiment 2	Positive cells counted 3x fields	98	78	68	146	284	265	411	301	96	34	13
		All cells counted 3x fields	1958	1982	1354	1126	1860	1603	1716	1814	2056	1738	1916
mN2[73bp]-tk-EGFP	Average	Mean percentage of GFP + cells	7.7	12.6	10.8	17.8	17.1	18.2	21.1	19.8	11.4	2.8	1.0
		SD of percentage of GFP + cells	4.2	4.1	1.3	0.7	3.8	11.7	9.6	3.1	0.2	0.8	1.1
	Experiment1	Positive cells counted 3x fields	160	109	139	126	313	94	411	272	140	71	27
		All cells counted 3x fields	1733	1324	1830	773	2284	981	2846	1607	1434	1911	1380
	Experiment 2	Positive cells counted 3x fields	114	286	168	337	485	211	185	207	169	35	5
		All cells counted 3x fields	2146	1610	1390	1904	2143	938	902	1029	1478	2196	2112
[mN2]-176bp-tk-EGFP	Average	Mean percentage of GFP + cells	0.8	0.7	0.5	1.3	0.4	2.7	1.1	0.7	1.4	0.6	0.2
		SD of percentage of GFP + cells	0.2	0.4	0.2	0.4	0.5	1.2	0.2	1.1	1.7	0.4	0.2
	Experiment1	Positive cells counted 3x fields	17	11	9	6	14	22	14	11	16	4	5
		All cells counted 3x fields	2495	1782	1631	1766	2185	1160	1600	912	1008	810	1572
	Experiment 2	Positive cells counted 3x fields	8	9	7	25	0	22	8	0	6	4	0
		All cells counted 3x fields	1317	2332	2290	2506	1518	1337	1076	879	2321	1804	814
[mN2]-73bp-tk-EGFP	Average	Mean percentage of GFP + cells	0.9	0.3	0.1	0.6	0.2	0.0	0.1	0.2	0.4	0.4	0.3
		SD of percentage of GFP + cells	1.0	0.4	0.0	0.5	0.2	0.1	0.1	0.4	0.2	0.0	0.0
	Experiment1	Positive cells counted 3x fields	4	7	2	4	7	2	2	0	4	11	3
		All cells counted 3x fields	1770	1518	1948	1197	2379	1019	2678	1300	2009	2015	2299
	Experiment 2	Positive cells counted 3x fields	10	0	2	4	2	0	3	10	5	8	4
		All cells counted 3x fields	1187	1876	2185	1147	2090	2021	1000	1691	1658	1852	1444

Table 15: Analysis of variance in the activity of the N2 enhancer and subregions in cES cells

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
PCAB	Between Groups	1767.111	10	176.711	1.302	0.252
	Within Groups	7463.506	55	135.7		
	Total	9230.618	65			
MN2_FULL	Between Groups	4050.107	10	405.011	8.018	>0.001
	Within Groups	2778.366	55	50.516		
	Total	6828.473	65			
MN2_176	Between Groups	4112.111	10	411.211	7.068	>0.001
	Within Groups	3200.011	55	58.182		
	Total	7312.122	65			
MN2_73	Between Groups	2777.772	10	277.777	4.275	>0.001
	Within Groups	3573.355	55	64.97		
	Total	6351.127	65			
MN2176Del	Between Groups	28.775	10	2.878	1.742	0.094
	Within Groups	90.835	55	1.652		
	Total	119.61	65			
MN273Del	Between Groups	3.449	10	0.345	0.946	0.499
	Within Groups	20.045	55	0.364		
	Total	23.494	65			
CN2_FULL	Between Groups	3608.942	10	360.894	10.18	>0.001
	Within Groups	1949.865	55	35.452		
	Total	5558.807	65			

Table 16: Paired analysis of activity of the mouse N2 enhancer and subregions in c ES cells.

Paired Samples Test										
Day of time course	Plasmids compared		Paired Differences					t	df	Sig. (2-tailed)
			Mean	SD	Std. Error Mean	95% Confidence Interval of the Difference				
						Lower	Upper			
Day 0	Pair 1	MN2_FULL - CN2_FULL	-2.41	8.86	3.61	-11.71	6.88	-0.66	5	0.535
	Pair 2	MN2_FULL - MN2_176	4.37	6.98	2.85	-2.95	11.71	1.53	5	0.185
	Pair 3	MN2_FULL - MN2_73	5.35	10.63	4.34	-5.80	16.51	1.23	5	0.272
	Pair 4	MN2_FULL - MN2176Del	12.28	7.78	3.17	4.11	20.44	3.86	5	0.012*
	Pair 5	MN2_FULL - MN273_Del	12.20	7.77	3.17	4.05	20.36	3.84	5	0.012*
Day 5	Pair 1	MN2_FULL - CHKN2FUL	-2.188	4.83	1.97	-7.25	2.88	-1.10	5	0.318
	Pair 2	MN2_FULL - MN2_176	3.77	8.09	3.30	-4.72	12.26	1.14	5	0.306
	Pair 3	MN2_FULL - MN2_73	4.99	14.28	5.83	-9.99	19.98	0.85	5	0.431
	Pair 4	MN2_FULL - MN2NO176	20.48	6.48	2.64	13.68	27.27	7.74	5	0.001*
	Pair 5	MN2_FULL - MN2NO73	23.13	6.79	2.77	16.00	30.26	8.34	5	<0.001*
Day 10	Pair 1	MN2_FULL - CHKN2FUL	-0.43	0.81	0.33	-1.29	0.41	-1.31	5	0.245
	Pair 2	MN2_FULL - MN2_176	-0.08	0.41	0.16	-0.51	0.34	-0.50	5	0.637
	Pair 3	MN2_FULL - MN2_73	-0.63	1.11	0.45	-1.80	0.54	-1.38	5	0.225
	Pair 4	MN2_FULL - MN2NO176	0.18	0.44	0.18	-0.28	0.64	1.00	5	0.36
	Pair 5	MN2_FULL - MN2NO73	0.07	0.62	0.25	-0.58	0.73	0.29	5	0.779

Figure 5.8: Activity of the N2 enhancer and its subregions during induced differentiation of cES cells

The activity of the N2 enhancer and its sub-regions in cES cells during the first ten days of a 21-day monolayer culture in N2B27 defined medium induced neuronal differentiation protocol. GFP was detected throughout the time course with the positive control pCAB-GFP indicating independence of the transfection efficiency from the time point (A1-A10 and B1-B10). The percentage of green cells detected following the transfection of the full-length mouse sequence of the N2 enhancer increased between day 4 and day 7 of the time course (C4, D4 and C7, D7 respectively). Similar changes were noted when the 176 bp and 73 bp core regions were used (E1-10, F1-10 and G1-10, H1-10 respectively). Throughout the time course, the activity of the N2 enhancer is negligible when these 176 bp and 73 bp subregions are deleted (I1-10, J1-10 and K1-10, L1-10 respectively) indicating the specificity of this activity to the 176 bp and 73 bp core regions of the N2 enhancer (Scale bar = 50μm).

Figure 5.9: Activity of the N2 enhancer and its subregions during induced differentiation of cES cells

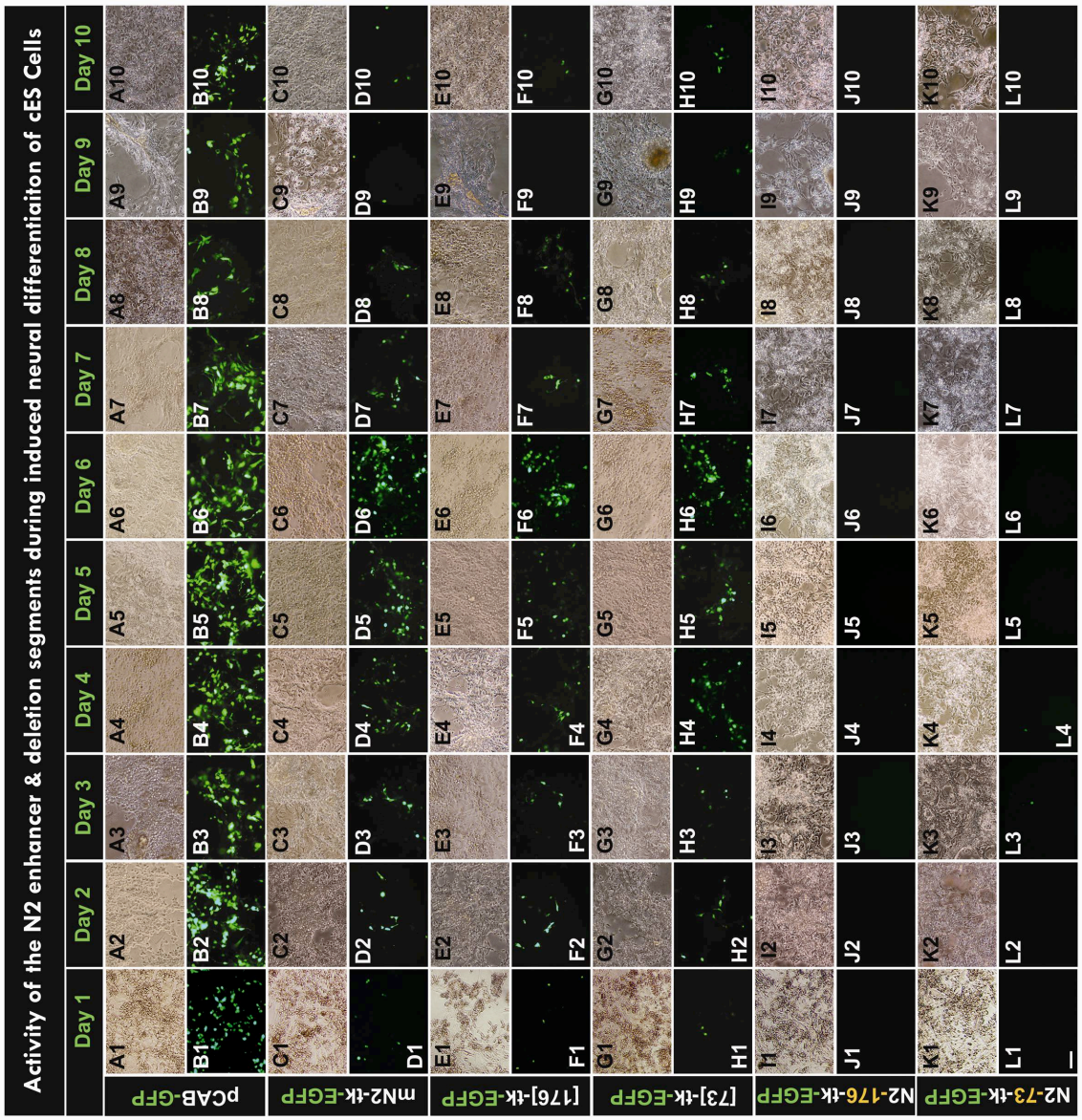


Figure 5.10: Quantification of activity of the N2 enhancer and its subregions during induced differentiation of cES cells.

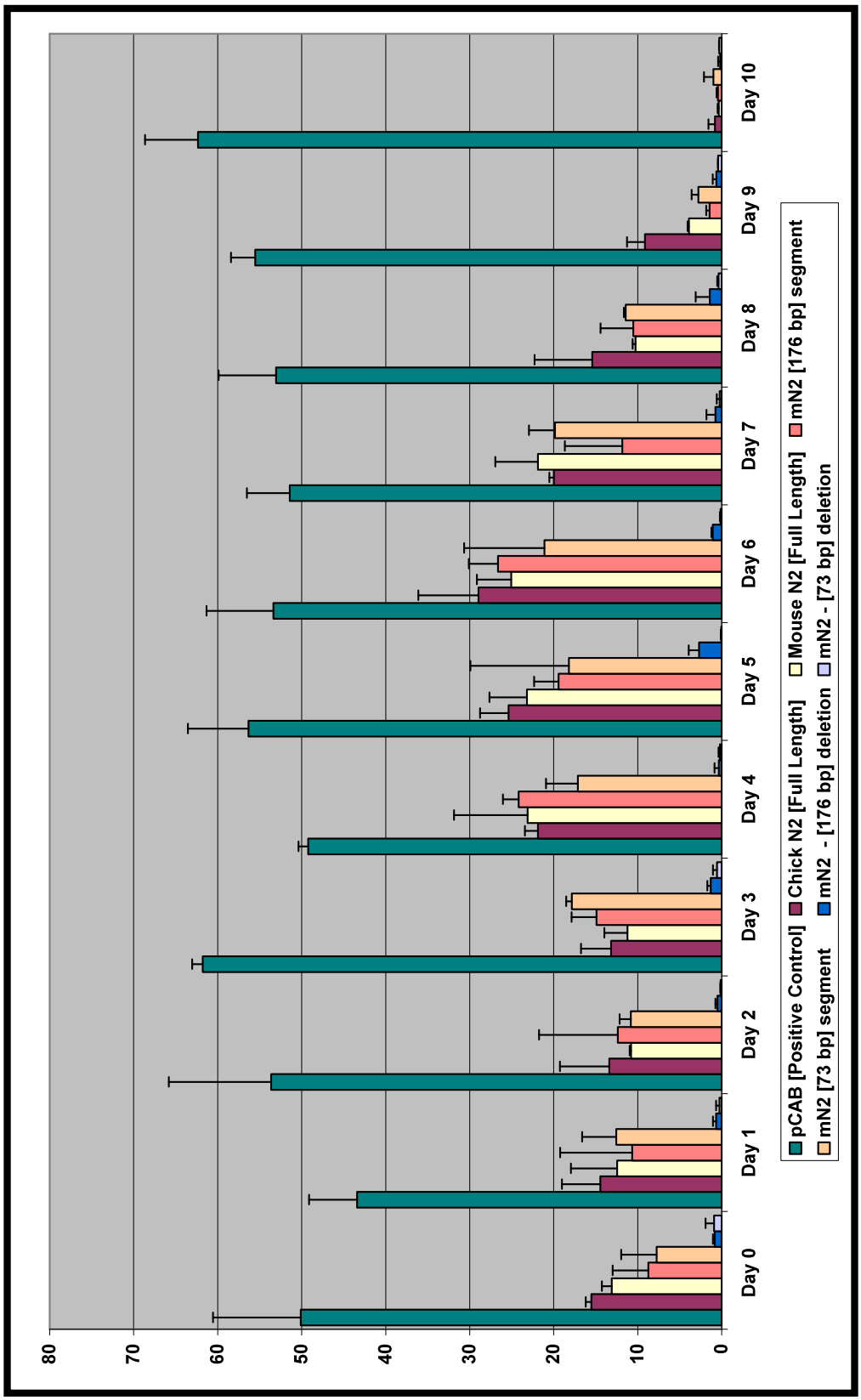
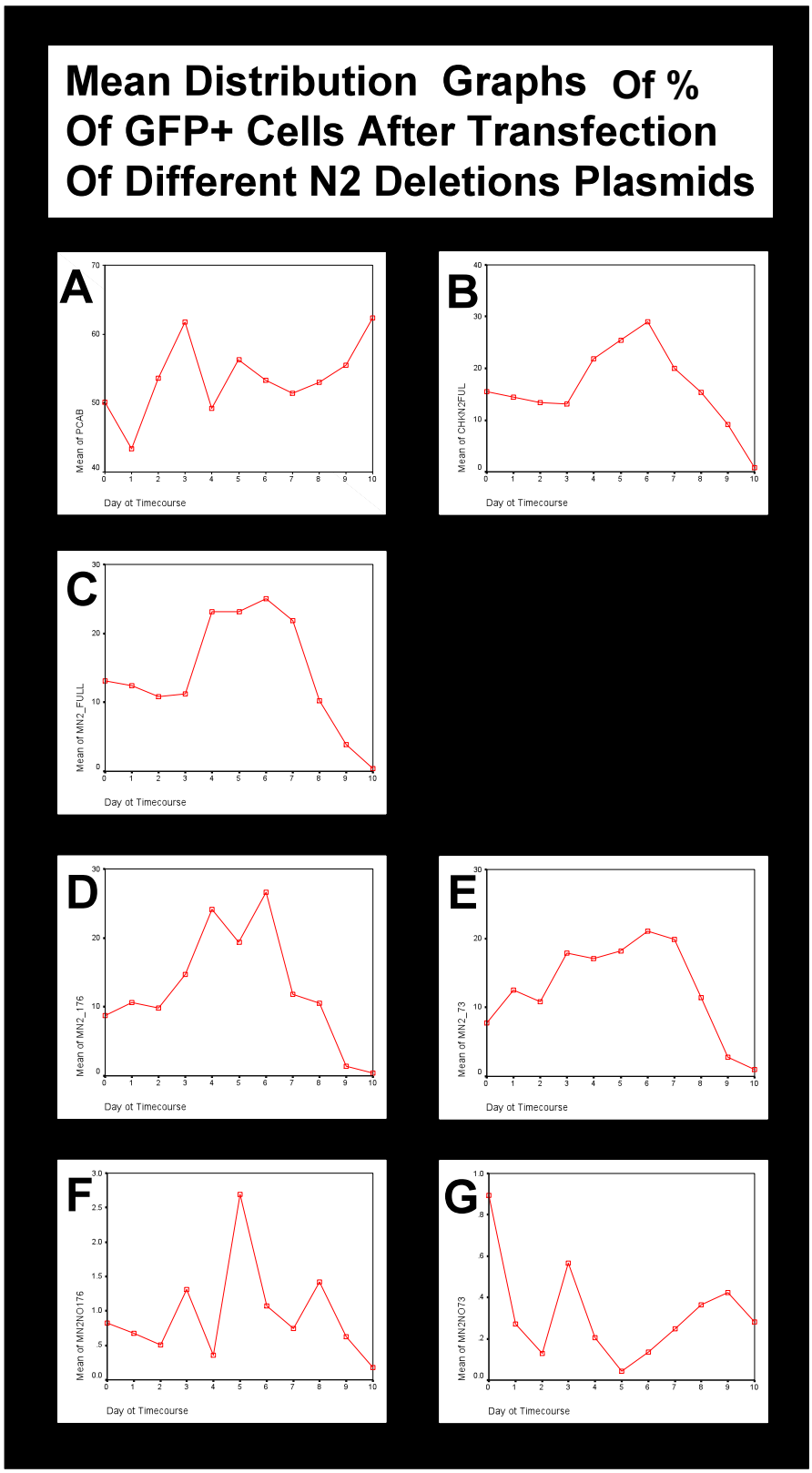


Figure 5.11: Patterns of activity of the N2 enhancer and its subregions during a 10 days time course of induced neural differentiation of cES cells

The mean percentages of green cells detected following the transfection of the N2 enhancer and its sub-deletions' tk-EGFP plasmids are plotted against time points of first 10 days of monolayer culture N2B27 induced neural differentiation protocol to compare patterns of activity of the N2 enhancer and its regulatory subregions under condition that induce neural differentiation. A: The positive control plasmid pCAB-GFP's enhancer activity changed during the time course ranging between 40% and 60% approximately. B: The activity of the chick N2 full length enhancer has a distinctive sigmoid pattern that shows its unchanged activity in the first third of the time course, increase activity in the second third of the time course and a late decrease in its activity in the last third. The activity of the mouse N2 enhancer (C) and its core 176 bp (D) and 73 bp (E) sub-regions have patterns which resemble the sigmoid pattern of the chick N2 full length enhancer (in shape and mean percentage changes) under these conditions. The activity of the N2 enhancer is reduced significantly when the 176 bp and 73 bp subregions are deleted (F and G respectively). This minimal activity has no resemblance in its pattern to neither that of the chick N2 full length or the mouse N2 full-length patterns.

Figure 5.11: Patterns of activity of the N2 enhancer and its subregions during a 10 days time course of induced neural differentiation of cES cells



5.3.3. Sox2 mRNA expression correlates N2 activity during induced neural differentiation

No significant fluctuation in transfection efficiency was observed during the time course (Figure 5.12: A0-A10 and B0-B10) also see Table 15. On the other hand, both the proportion of cells activating the N2 enhancer (Figure 5.12: C0-C10; D0-D10 and Figure 5.13) and the expression of *Sox2* mRNA (Figure 5.12: E0-E10 & F0-F10 and Figure 5.13) varied between days. In the first 3 days of the time course *Sox2* mRNA is downregulated (Figure 5.12: E0, E3 & F0, F3). During the same period the proportion of cells expressing N2 does not seem to change (Figure 5.12: C0, C3). On day 4 *Sox2* mRNA starts to increase (Figure 5.12: E4, F4 and Figure 5.13). This is accompanied by increased activity of the N2 enhancer (Figure 5.12: C4, D4 and Figure 5.13).

In the second half of the time course, both *Sox2* expression and the number of cells activating the N2 enhancer first increase, and then decrease (Figure 5.12: C5-C10; D5-D10; E5-E10; F5-F10 and Figure 5.13). N2-enhancer expression peaks on the 6^{th} day (Figure 5.12: C6, D6 and Figure 5.13), one day earlier than the peak expression of *Sox2* mRNA (Figure 5.12: E7, F7 and Figure 5.13).

A correlation calculation between Sox2 mRNA expression and the activity of the mN2 enhancer of Sox2 was conducted to investigate any association between the two during the time course. The test was done on days 0 – 10 of the time course with N=11 data pairs. The null hypothesis (no association) was rejected at ($p<0.05$).

Pearson's r was 0.665. With 9 degrees of freedom (N-2) and alpha level of 0.05 the null hypothesis was rejected. The critical value of r for these two parameters was 0.521. We conclude that there is a significant positive correlation between the activity of N2 and the mRNA expression of Sox2 in cESCs induced to neural differentiate in vitro.

Figure 5.12: Comparison between dynamic changes in Sox2 mRNA expression and the activity of the N2 enhancer during induced neural differentiation of cES cells

A 10 days time course of cES cells induced neural differentiation was used to compare the activity of the N2 enhancer (top four panels) to that of the mRNA expression of *Sox2* (bottom two panels). First, the activity of the positive control plasmid pCAB-GFP is shown in the top panel confirming good transfection efficiency on all points of the time course (A01-10 and B0-10). The activity of the N2 enhancer in cES cells increased in the 1st half of a 10-days time course of induced neural differentiation (D0-D6) and decreased in the 2nd half (D6-D10). This variability was not related to transfection efficiency assessed by the positive control plasmid shown in the top panel. By ISH The number of cells expressing Sox2 mRNA in cESCs during a parallel time course under similar condition was noted to change with initial decrease in the 1st third of a 10-days time course (F0-F3); then an increase in the 2nd third peaking at day 7 (F4-F7); then another decrease in the last third (F8-F10). These changes in the N2 activity and Sox2 mRNA expression were quantified and found to be significantly correlated (See text)(Scale bar = 50μm)

Figure 5.12: Comparison between dynamic changes in Sox2 mRNA expression and the activity of the N2 enhancer during induced neural differentiation of cES cells

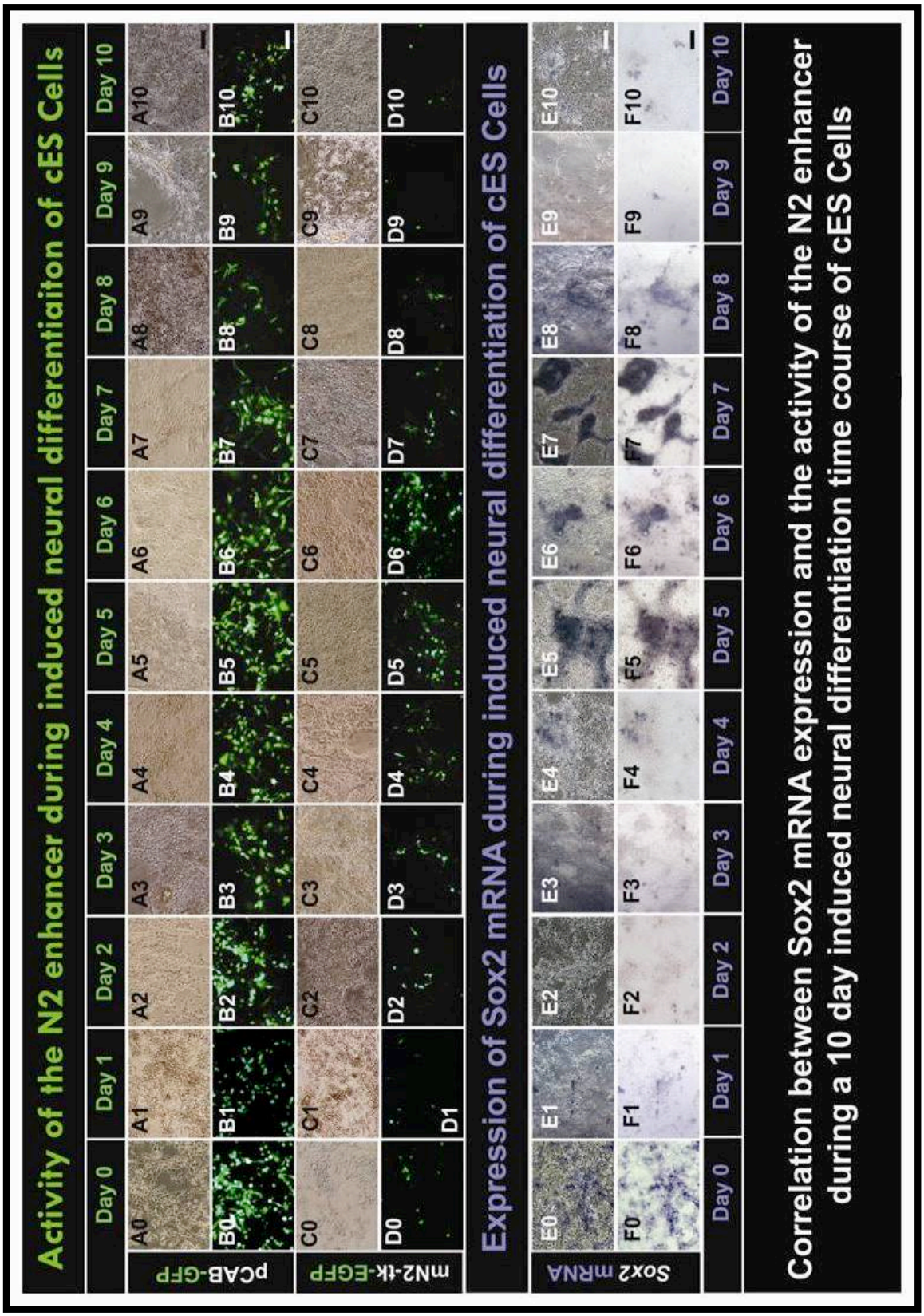
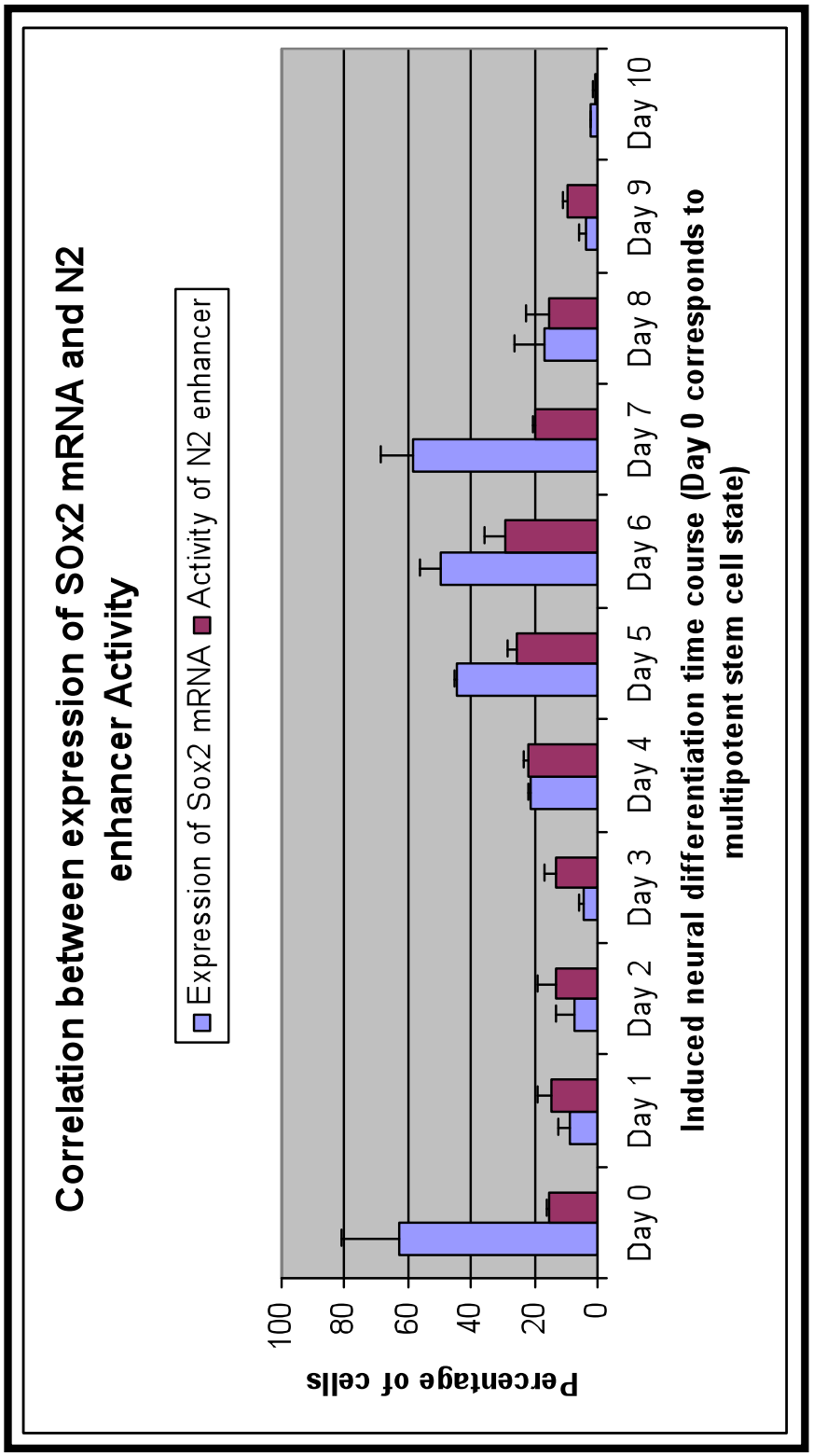


Figure 5.13: Dynamic changes in Sox2 mRNA expression correlates with the activity of the N2 enhancer during induced neural differentiation of cES cells

This chart shows the correlation between Sox2 mRNA expression and N2 activity during a 10 days time course. By comparing the percentage of cells expressing Sox2 mRNA (blue) and those with active N2enhancer (red) from 2 independent experiments we find that expression of Sox2 mRNA is significantly correlated with the activity of N2 enhancer (r = 0.665) $p < 0.05$

Figure 5.13: Dynamic changes in Sox2 mRNA expression correlates with the activity of the N2 enhancer during induced neural differentiation of cES cells



5.4. Discussion

5.4.1. Time course of changes in gene expression during induced neural differentiation of cES cells

A time course of the changes in the expression of 10 markers was undertaken for cES cells in the proliferating state (t=0) and on each day thereafter following removal of conditions that support proliferation and which induce differentiation. In this case N2B27 medium was used in the presence of 1% fetal calf serum but without the addition of retinoic acid as described elsewhere in this thesis.

In general, it was found that proliferating ES cells express the proliferating 'stem' state markers *Sox2*, *Nanog*, *ERNI and Oct4*. The expression of early neural markers (including *Sox2*, *Sox3* and *Otx2*) starts to increase in the second half of the time course. The marker of the most mature neural plate used in this set was *Sox1*, which starts to be expressed on day 5. At the same time some cells acquire expression of the mesendodermal marker *Brachyury* (Kispert et al., 1995) from day 6, but no expression of the early endodermal marker (Kimura et al., 2006) was observed at any stage during the time course. Thus, N2B27 under these conditions induces differentiation into neural cells; at least a proportion of cells also differentiate into mesoderm or mesendoderm, judged by the expression of *Brachyury*.

5.4.2. Activity of the N2 enhancer and its sub-regions in cES cells

In chick embryonic stem cells, N2 was found to be the Sox2 enhancer with highest activity in the proliferative state (Chapter 3). N2 is also the first enhancer to be activated in the embryo after gastrulation, when Sox2 expression begins in the forming neural plate (Collignon et al., 1996; Rex et al., 1997b; Uchikawa et al., 2003; Uwanogho et al., 1995).

When cES cells were induced to differentiate in vitro, N2 activity increased, as did expression of *Sox2* mRNA. As in the embryo where *Sox2* expression is downregulated between pre-gastrulation stages and the end of gastrulation (stages 4-5) when it reappears in the neural plate, there appears to be downregulation of *Sox2* with no loss of activity of the N2 enhancer.

This could be partially due to timing of transfection and accumulation of the GFP protein. As N2 is active in proliferating cES cells, transfection of the plasmid in the proliferating phase at the beginning of the time course could transiently lead to GFP resultant from such activity. Similarly, as fluorescent cells are assayed 36-48 hours following transfection, it is possible that some of the GFP detected would be resulting from this second increase in activity of the N2 enhancer. ISH detects mRNA present at the time of fixing and provide a snapshot of the state of gene expression during such time. On contrast, the activity of the enhancer is accumulative and provides information on several time points.

The increase in the N2 activity, which seems to accompany the upregulation of Sox2 in the second half of the time course between the proliferating stage and the acquisition of neural fate, occurs about 5 days into the time-course of induced differentiation. These findings suggest that N2 contains the information to direct expression of *Sox2* both in embryonic stem cells in the proliferating state and during neural differentiation, as well as in the early neural plate of the embryo.

Following on from the original paper describing the various enhancers of *Sox2* (Uchikawa et al., 2003), Kondoh's laboratory identified two highly conserved sub-regions of the N2 enhancer (Iwafuchi et al., 2008). Both the full N2 enhancer (550 bp) and its two sub-regions were found to be conserved between mouse and chick in vivo. In the latter study, the full mouse N2 and its two sub-regions (named N2-176 and N2-73) were found to direct expression of a reporter in the *Sox2* expression domain at stage 4+ when coupled with a minimal promoter and electroporated into primitive streak stage chick embryos

(Iwafuchi et al., 2008). Furthermore, the full length of mouse N2 sequence, as reported in the chick in chapter 3, is active in mouse embryonic stem cells in their proliferating state (Catena et al., 2004). The core sub-regions of the N2 enhancer had similar activity to the full length N2 (Catena et al., 2004).

Sox2 is expressed in the Inner Cell Mass of early mouse embryos (Avilion et al., 2003) as well as in developing neural tissue (Zappone et al., 2000). This expression appears to be driven by a non-coding region 5' of the *Sox2* locus which can direct expression to the inner cell mass as well as later to the anterior neural plate (Zappone et al., 2000). This non-coding region is located between 3.3 and 5.7 kb upstream of the *Sox2* coding sequence. A sequence homologous to the chick N2 enhancer is found between 3.8 and 4.3 kb upstream of the *Sox2* reading frame and therefore is entirely contained within the 5' region identified by Zappone et al. (2000) (Uchikawa et al., 2003). In vitro, this 5' region of *Sox2* was active in neural progenitor cells derived from anterior neural tube but not from the spinal cord (Zappone et al., 2000). This very regulatory element was reported later by the same group to drive expression of *Sox2* to mouse ES cells (Catena et al., 2004). Similar findings were also reported for mouse ES cells by Kondoh's group using the 550 bp N2 enhancer and its sub-regions (Iwafuchi et al., 2008).

Catena et al (2004) further analysed the 5' regulatory sequence and identified a core sub-region with two POU binding sites that can recapitulate the activity of the entire original 5' region. Mutation of these POU sites rendered this core region inactive for both 'neural progenitors' and 'multipotent' states of ES cells (Catena et al., 2004). Further analysis by Chromatin Immunoprecipitation reported different members of the POU family of transcription factors are associated with this region in the two states. In the 'stem' state PouV is bound to these sites, whereas in the 'neural' state these sites are occupied by Brn1 and Brn2 (Catena et al., 2004). Both Brn1 and Brn2 (also known as Qin in the chick embryo) are expressed in the early anterior neural plate of late primitive streak and neurulating stage embryos (Witta et al., 1995) .

In the chick, Kondoh's group also analyzed the activity of the N2 enhancer and identified two sub-regions: the first is 176bp and the second is a 73 bp core region is contained within the former (Iwafuchi et al., 2008) (see Appendix 1). The two POU sites described by Catena et al. (2004) are contained within the smaller 73bp sub-region. Kondoh's group also confirmed the finding of Catena et al (albeit using Electrophoretic Mobility Shift Analysis rather than Chromatin Immunoprecipitation) that PouV is associated with the 73bp core region in mouse ES cells in the proliferating state (Iwafuchi et al., 2008). However for the neural plate they found that the activity of the core region depends on binding of a different transcription factor to the 73 bp sequence: Otx2. All of these studies indicate that the activity of Sox2 in cultured mouse embryonic cells in the 'stem' and 'neural' states, as well as in Sox2 expressing mouse ICM cells and later in the early forebrain domain all rely on the 73 bp N2 core region.

The results reported in this chapter support the findings from the mouse: the enhancer N2 has the highest activity in proliferating chick ES cells. They also confirm that the smaller 176 and 73 bp core regions are sufficient to direct expression of a reporter. Furthermore, and for the first time, this chapter reports that blastoderm-derived cES cells induced to differentiate in vitro display an increase in activity of the N2 enhancer (and both its sub-regions), correlated with the increase in the number of cells expressing *Sox2* mRNA. This suggests that the 73bp core region of the N2 enhancer contains sufficient information to direct expression of *Sox2* in both the 'stem' state and during induced neural differentiation state as well as in the anterior neural plate of the embryo.

Analysis of the N2 enhancer identified 66 conserved potential transcription factor binding sites, which can be reduced to 35 highly matching sites (see Appendix). Within the shorter sub-regions of 176bp and 73 bp the list drops to 18 and 7 sites, respectively. Mutational analysis on these regions is required to determine which of these are required for the activity of these core enhancer regions in vivo and in vitro. A similar approach has been used to

show that factors associated with Wnt and FGF signalling synergistically activate the N1 (posterior neural plate) enhancer (Takemoto et al., 2006).

A recent functional study in the chick identified another mechanism regulating *Sox2* expression in the early neural plate (Papanayotou et al., 2008). In this study, interactions between three coiled-coil domain proteins (ERNI, Geminin, and BERT), the heterochromatin proteins HP1α and HP1γ (transcriptional repressors) and the chromatin-remodelling enzyme Brm (as activator) control the timing of activation of the N2 enhancer. Chick ES cells provide a platform where factors identified in the embryo could be tested to explore whether the same factors will be interpreted in the same way by the ES cells.

General Discussion

The Sox2 gene has been implicated as a key factor in several important developmental processes (Miyagi et al., 2009; Zhao et al., 2004). In the developing embryo, *Sox2* is first expressed in a broad domain of the pre-primitive-streak stage embryo in both mouse (Avilion et al., 2003; Wood and Episkopou, 1999; Zhappone et al., 2000) and chick (Bertocchini, Boast and Stern, in preparation). Then, expression is downregulated before reappearing in the developing neural plate soon after the end of gastrulation (Kondoh et al., 2004; Papanayotou et al., 2008; Rex et al., 1997b; Uchikawa et al., 2003; Uwanogho et al., 1995). Thereafter it remains expressed almost throughout the central nervous system and eventually ending in the residual neural progenitors of adult ventricular zone as well as in a number of domains outside the CNS, including sensory placodes and other sites (bu-Elmagd et al., 2001; Ishii et al., 1998; Le et al., 2002; Rex et al., 1994; Rex et al., 1997b; Uwanogho et al., 1995). The pioneering work of Hisato Kondoh's group demonstrated that at different sites, Sox2 expression is controlled by distinct enhancers. They identified 10 different enhancers within 25 highly conserved sequences, situated both upstream and downstream of the single coding exon of *Sox2* (Kondoh et al., 2004; Uchikawa et al., 2003).

In the neural plate, *Sox2* expression begins in the most anterior domain, encompassing the forebrain, midbrain and anterior hindbrain to the level of the otic vesicle (Papanayotou et al., 2008; Rex et al., 1994; Rex et al., 1997b; Uwanogho et al., 1995). This expression is regulated by the N2 enhancer, located upstream of the Sox2 coding region in chick, mouse and human (Uchikawa et al., 2003). The remainder of the CNS (posterior hindbrain and spinal cord) derives from a much smaller region, two small triangles of epiblast on either side of Hensen's node that have been termed the "stem zone" (Delfino-Machin et al., 2005). A different enhancer, N1, drives *Sox2* expression in this domain (Uchikawa et al., 2003).

Sox2 is also expressed in mouse (Maruyama et al., 2005; Masui et al., 2007) and human (Card et al., 2008; Fong et al., 2008) embryonic stem cells and it is usually considered one of the genes whose expression defines pluripotency and/or the capacity for self-renewal (Rizzino, 2009; Wang et al., 2007). Consistent with this, *Sox2* is also expressed in the cells of the inner cell mass of early mouse embryos, from which mouse ES cells are derived. In contrast, original reports on *Sox2* expression in chick embryos did not find expression prior to the late primitive streak stage (Rex et al., 1997b; Uwanogho et al., 1995). A recent re-examination of this issue did reveal a very early, transient phase of *Sox2* expression in the chick embryo before gastrulation, at stage XIII (Eyal-Giladi and Kochav, 1976) as well as its expression in chick embryonic stem cells, derived from pre-primitive-streak stage embryos (F. Bertocchini, S. Boast and C.D. Stern, in preparation). These findings suggest that the avian and mammalian systems may not differ in their expression of SoxB1 class genes as much as was previously thought.

ES cells isolated from the chick embryo share many ES cell features identified in their mammalian counterparts including the capacity for self-renewal and their ability to contribute to all somatic cell types (Lavial and Pain, 2010; Pain et al., 1996; Pain et al., 1999) and the expression of markers of pluripotency and/or self-renewal, such as PouV (the chick homologue of Oct3/4) and cNanog (Lavial et al., 2007) in addition to *Sox2*. However unlike their mouse counterparts, chick embryonic stem cells derived from the early blastdoderm appear to be unable to contribute to the germ line (Lavial and Pain, 2010; Pain et al., 1999). This is not a property of avian cells in general because it is possible to derive self-renewing cell lines from primordial germ cells that do have the ability to contribute to both somatic and germ lines (McGrew et al., 2004; Naito et al., 1994; Petitte et al., 1990). These primordial-germ-cell-derived (PG) cell lines also express markers of self-renewal and pluripotency including *PouV*, *Nanog* and *Sox2* in addition to the germ cell markers *Vasa* and *Dazl* (Lavial et al., 2009; Montono et al., 2008; Tsunekawa et al., 2000 and S. Intarapat and Stern, unpublished observations). These observations suggest that the so-called chick embryonic stem cells are not strictly equivalent to mouse ES cells (which can contribute to both somatic and germ

lineages), but rather resemble the stem cell lines that can be derived from the epiblast of slightly later mouse embryos, known as Epiblast Stem cells whose potential is restricted to somatic descendants (Brons et al., 2007).

Hisato Kondoh's laboratory identified enhancers directing *Sox2* expression in embryonic tissues, but since chick ES cells were not known to express this gene, the enhancers responsible had not been sought. This was one of the aims of this project. The starting point is the hypothesis that the expression of *Sox2* in these cells is governed by mechanisms that are conserved between species. A recent study identified a region of non-coding DNA that is sufficient to drive *Sox2* expression mouse ES cells (Catena et al., 2004; Zappone et al., 2000). Cross-species comparisons and genome alignments reveal that this region contains within it the putative mouse homologue of the chick N2 enhancer identified by Uchikawa et al. (2003). For this reason, this work started by exploring whether the chick N2 enhancer has similar properties in chick ES cells, using constructs containing a minimal Tk promoter downstream of the enhancer region to be tested, driving expression of GFP as a fluorescent reporter. Constructs containing each of the *Sox2* conserved non-coding blocks were kindly provided by Professor Hisato Kondoh. These were transfected into three different established lines of chick ES cells.

Following transfection of these 26 different constructs only that containing N2 was found to have significant activity in proliferating chick ES cells. The enhancer N2 is contained within the region upstream of Sox2 which was found to be active in mouse ES cells (Catena et al., 2004) and which can also drive expression in mouse neural progenitors (Zappone et al., 2000) and, in the chick, in a wide anterior domain of the neural plate destined to form the future brain (Uchikawa et al., 2003). The N2 enhancer was further dissected into sub-regions, which were studied for their activity in chick ES cells. In agreement with findings from the Kondoh laboratory using mouse ES cells, an inner "core" region of 73 bp contained within a larger 176 bp domain, are both sufficient and necessary for expression in chick ES cells. These regions contain several locations corresponding to putative POU binding sequences

(Catena et al., 2004; Iwafuchi et al., 2008; Uchikawa et al., 2003) (also see Appendix 1).

Since the N2 enhancer appears to be responsible for directing expression of *Sox2* in both proliferating ES cells and in the forming anterior neural plate, what are the dynamics of the changes in its activity during the process of differentiation in vivo and in vitro? In vivo, the early expression phase appears to occur at stage XIII of Eyal-Giladi & Kochav (1976) (Bertocchini, Boast & Stern, in preparation) and the late phase starts at Hamburger & Hamilton (1951) stage 4+, with no express in the intervening period. (Kondoh et al., 2004; Papanayotou et al., 2008; Rex et al.,1997b; Uchikawa et al., 2003; Uwanogho et al., 1995). To examine the dynamics of *Sox2* expression and the activity of the enhancers directing this during induced neural differentiation of chick ES cells, it was necessary first to design a reproducible protocol for obtaining neural differentiation of chick ES cells in vitro. Several methods have been reported for mouse ES cells (Bain et al., 1995; Dani et al., 1997; Ying and Smith, 2003) but these have not systematically be tested in the chick.

The next part of the project therefore moved on to compare two methods for inducing chick ES cells to acquire a neural fate. One was an Embryoid Body (EB) -based protocol (based on Bain et al., 1995) and the other a monolayer culture in defined medium containing differentiation-inducing factors (based on Ying et al., 2003; Ying and Smith, 2003). In both cases cES cells differentiated into neuron-like cells which express the neurofilament-associated antigen 3A10 (Furley et al., 1990; Storey et al., 1992;Yamada et al., 1991) and the neuronal class 3 β-tubulin antigen TUJ-I (Ferreira and Caceres, 1992; Maurer et al., 2007; Miura and Kameda, 2001; Scott et al., 1990). However the monolayer culture method was found to be more reliable and more effective.

Next, the gene expression profile of 10 genes that mark different cell types and stages of neural plate development in the embryo was assayed by in situ hybridisation in proliferating ES cells and over a 10-day time-course during induced neural differentiation. We found that proliferating cES cells have a gene expression profile which is similar to that of proliferating mouse and

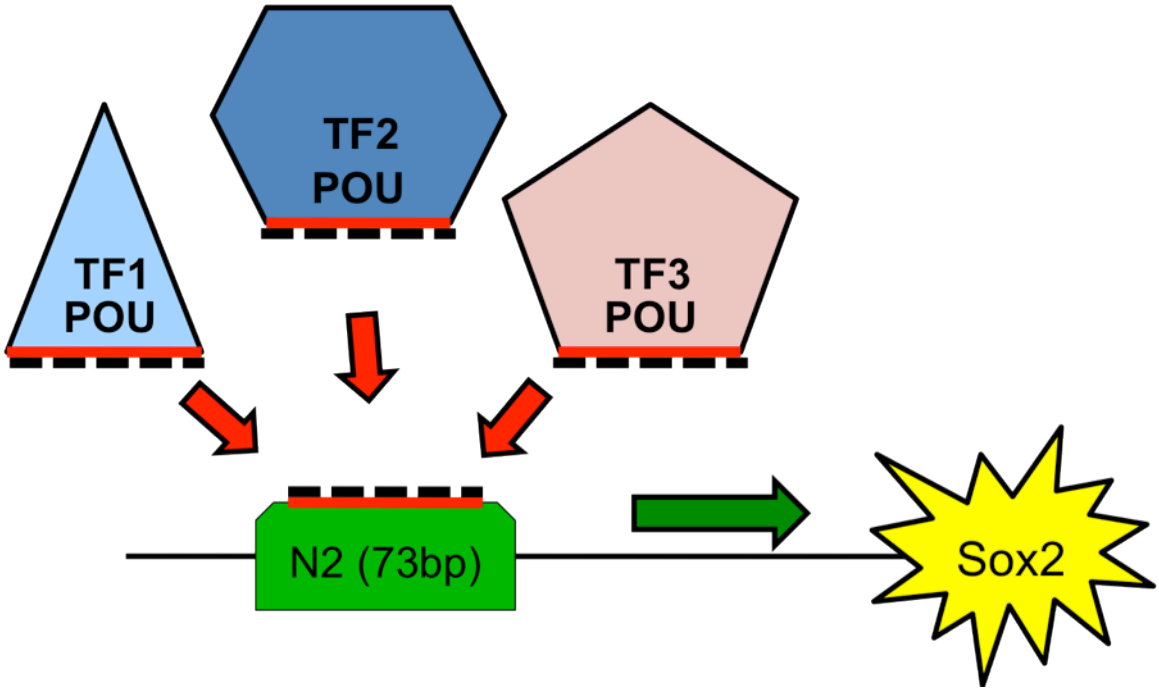
human ES cells (Adewumi et al., 2007; Smith, 2001). Marked upregulation of the expression of SoxB1 genes *Sox2* and *Sox1* was found to occur in the second half of the time course.

We hypothesize that this upregulation of Sox2 in the second half of the induced neural differentiation time course is comparable with the second phase of Sox2 expression in the embryo, when the neural plate starts to become distinct at stage 4^{+}-5 (Kondoh et al., 2004; Papanayotou et al., 2008; Rex et al.,1997b; Uchikawa et al., 2003; Uwanogho et al., 1995). When the activity of the N2 enhancer was examined during the 10-day time-course, a marked increase in the activity of the N2 enhancer was found to correlate with the upregulation of *Sox2* mRNA expression observed in the second half of the time course. Taken together, these findings indicate that the N2 enhancer is conserved in the chick and mouse not only in the DNA sequence, but also in its regulatory functions in both cultured ES cells and in the embryo.

The sequence of the N2 enhancer and its essential core regions is highly conserved between mammals and chicken (Kondoh et al., 2004;Uchikawa et al., 2003) and (Appendix 1). In the core sequence of 73 bp, two POU binding sites are conserved. These sites are both necessary and sufficient for expression in mouse ES cells in both the proliferating and induced neural states (Catena et al., 2004 and this thesis). These POU sites have been reported to be bound by Oct3/4 in ES cells in the proliferating state (Catena et al., 2004; Nichols et al., 1998; Rosner et al., 1990; Tanaka et al., 2004), whereas the related POU factors Brn1 and Brn2 may bind to the same sites in differentiating neural cells (Catena et al., 2004; Eisen et al., 1995; Josephson et al., 1998; Matsuo-Takasaki et al., 1999; Witta et al., 1995). In the early chick neural plate the activity of this core region was similarly attributed to POU factors as well to Otx2 (Iwafuchi et al., 2008). These findings suggest that the essential regions (73 bp core of a larger 176bp essential region) of the N2 enhancer of Sox2 contain the instructions required for expression in cES cells in the growing phase and during neural differentiation, as well as in embryos before gastrulation and at the early neural plate stage.

In conclusion, this study revealed that the machinery controlling *Sox2* expression in chick ES cells in their undifferentiated, self-renewing state are comparable to those controlling it pre-gastrulating embryos as well as in the later phase of early neural plate formation in vivo and during induced neural differentiation of ES cells. A single enhancer region, N2 (and specifically the 73 bp core of an important sub-region of 176 bp) is necessary and sufficient to account for expression in all four of these situations. This is to some extent a surprising finding especially because 9 other enhancers have been identified that contribute to controlling *Sox2* expression in vivo in a number of different sites, including 4 enhancers in addition to N2 which direct expression to the central nervous system (Kondoh et al., 2004; Uchikawa et al., 2003) It seems that for these earliest stages of development and during ES cell growth and differentiation, the main mechanism regulating the changes in the expression of this gene relies on differential expression of the transcription factors that bind to this single enhancer, which appear to be different members of the POU family.

Figure 30: Thesis Diagram



A single enhancer region, N2 (and specifically the 73 bp core of an important sub-region of 176 bp) is necessary and sufficient to account for expression of *Sox2* in chick ES cells cultured *in vitro* iunder conditions that maintains their undifferentiated, self-renewing state as well during their induced neural differentiation. This function of the N2 enhancer core region is comparable to that controlling *Sox2* expression *in vivo* during the early phase of neural plate formation. This thesis suggests that for embryonic development and during ES cell growth and differentiation, the main mechanism regulating the changes in the expression of *Sox2* relies on differential expression of the transcription factors that bind to this single enhancer, which appear to be different members of the POU family

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APPENDIX I

1] Inter-species comparison of the N2 enhancer sequence

>N2 Chick
GAGGAAATTTCTCTGTACGTTTTCGTCTTCGGCTTTTTTTTTTTTA
ACGTAATGAAATTAAAACTTTTGGAGCTCAGAGTTGACATTTTGCGGAAA
ATTGAGTTATCAAGGCAGTAATTATTTCACAGGGAGATAAAACTCTCATA
GCCCTAACTGTCAAATAGGGCCCTTTTCAGATTTTAATTACAAAATAAAA
TTAGTCTGCTCTTCCTCGGAAGGGTTTGTGAGTGGCTAAACAGAGCTTTC
CCCAATACTGGTGGTCGTCAAACTCTGCTAATTAGCAATGCTGAGAAAT
TCCAGTTAACAAGGACATTCTCTAAGTCTCTGCAGGTTCCCTGCCGTTCG
CCTTCATTTCCATAAGAAGATTAAGAGTGGAGGGGAACACACTCAAATGC
AGATGCAGAAAAGAAGCGTTTTTAACAAGCATCATAATAGTAAGATGCT
TGGCTAGTTCTCACCTAATTAACTGCAAGTTAAACCTCTATTTGCAGCTAA
GGACAAAAAATGGAGCTGCAATCTTCCATCTCCACAAGAC

>N2 Mouse
TAGAAAGCCTTTCTGTACATTTTCTCTTATTTTTCTTGCTACTTTTCCTT
ATGTAATGAAATTAAAACTTTTGGA ACCCACAGTTGACATTTTTCAGAAA
ATTGAGTTATCAAGGCAGTAATTATTTCTCGGGGAGATAAAACTCTCATA
GCCCTAACTGTCAAATAGGGCCCTTTTCAGATTTTAATTACAAAATAAAA
TTAGTCTGCTCTTCCTCGGAATGGTTGGCGAGTGGTTAAACAGAGCTTTC
CCCCAATACTGGTGGTCGTCAAACTCTGCTAATTAGCAATGCTGAGAAAT
TCCAGTTAACAAGGGCATTCTCCGAGACTCTGCAGGTCCCCTGCCGTTC
GCCTTCATTTCCATAAGGAGATTAGGAGAGGAGGGGAACCCACTCAAAT
GCAGATGCAGGAGCGAAGCGTTTTTAACAAGCATCATAATAGTAAGATGC
TTGGCTAGTTCTCGCTAATTAACTGCAACTTAAACCTCTATTTGCAGCTAA
GAAGAAAAAAATAAGTCTACAGTCCGCACCTCCACAACAT

>N2 Human
TAGAAAACCTTTCTGTACATTTTCTTCTATTTTTCT CACTTTTTCCTT
ACATAATGAAATTAAAACTTTTGGAGCCTACAGTTGACATTTTTCAGAAA
ATTGAGTTATCAAGGCAGTAATTATTTCACGGGGAGATAAAACTCTCATA
GCCCTAACTGTCAAATAGGGCCCTTTTCAGATTTTAATTACAAAATAAAA
TTAGTCTGCTCTTCCTCAGAATGGTTTGTGAGTGGTTAAACAGAGCTTTC
CCCCAATACTGGTGGTCGTCAAACTCTGCTAATTAGCAATGCTGAGAAAT
TCCAGTTAACAAGGACATTCTCCAAGACTCTGCAGGTTCCCTGCCGTTCG
CCTTCATTTCCATAAGAAGATTAAGAGAGGAGGGGAACACACTCAAATGC
AGATGCAGAAAAGAAGCGTTTTTTAACAAGCATCATAATAGTAAGATGCT
TGGCTAGTTCTCACCTAATTAACTGCAAGTTAAACCTCTATTTGCAGCTAA
GAAGAAAAAATAAGTCTACAGTCCCCTGTCTCCACAAAAT

2] Analysis of sequence homology

Out of 530 bases of its full length in chick there are 477 and 490 base matches in their mouse and human counterparts rendering 90% and 93% sequence homology with these two species respectively. By By utilizing Mulan software (Loots and Ovcharenko, 2007) 66 multi-conserved transcription factor-binding sites (TFBS) were identified (Data not shown). The number of TFBS could be reduced to 35 when the search setting was increased to “High specificity” (Table Appx. 1 and Figure Appx. 1 &2).

Table Appx. 1: Conserved "highly specific" Transcription Factor Binding Sites (TFBS) identified in the sequence of *Sox2* N2 enhancer using Mulan software (Loots and Ovcharenko, 2007) .

No.	Transcription Factor Name	Location in the N2 sequence	Sequence
1	PAX	113-123	agTAATTATTt
2	VMYB	149-157	cctAACTGt
3	SRF	153-171	actgTCAAATAGGGccctt
4	SRF	155-168	tgTCAAATAGGGcc
5	SRF	156-173	gTCAAATAGGGccctttt
6	SRF	157-171	TCAAATAGGGccctt
7	PLZF	166-194	GCCCTTTTCAGATTTTAATTA
8	PF1	176-190	gatttTAATTacaaa
9	CEBP	183-194	aTTACAAAAtaa
10	CEBP	183-194	aTTACaaaataa
11	EVI1	186-194	ACAAAATAA
12	NFY	224-234	gtgAGTGGcta
13	NFY	225-237	tgAGTGGctaaac
14	GLI	255-262	tgGTGGtc
15	ATF	256-267	ggtggTCGTCAa
16	PAX3	261-273	TCGTCAAACtctg
17	CREB	262-267	CGTCAa
18	OCTI	268-279	acTCTGCTAATT
19	CHX10	270-283	tctGCTAATTAgca
20	CHX10	272-285	tgcTAATTAGCaat
21	NFKB	285-300	tgctgAGAAATTCCAg
22	AP1	285-297	tgcTGAGAAAttc
23	NFKAPPAB	290-299	AGAAATTCCa
24	ROSA1	301-313	ttaacaAGGACAt
25	OCT	348-360	tTCATTTCCATaa
26	OCTI	349-359	tcATTTCCATa
27	OCT	349-359	tcaTTTCCATa
28	VMAF	390-408	aaatGCAGATGCAGAaaag
29	WHN	408-418	gaaGCGTtttt
30	NFY	439-452	gaTGCTTGGCTagt
31	CAAT	441-452	tgCTTGGctagt
32	NFY	441-453	tgCTTGGctagtt
33	CHX10	455-468	tcaCCTAATTAact
34	CHX10	457-470	accTAATTAACtgc
35	AFP1	462-472	ATTAACTGCAA

Figure Appx. 1: Bioinformatics analysis of the N2 enhancer and identification of conserved Transcription Factors Binding Sites (TFBS).

Sequence homology of the N2 enhancer in the chick, mouse and man with the 176 bp and 73 bp core regions having the highest interspecies homology (100% for 73 bp between chick and man). The N2 enhancer encodes 66 conserved transcription factor-binding sites with 35 being with high specificity.

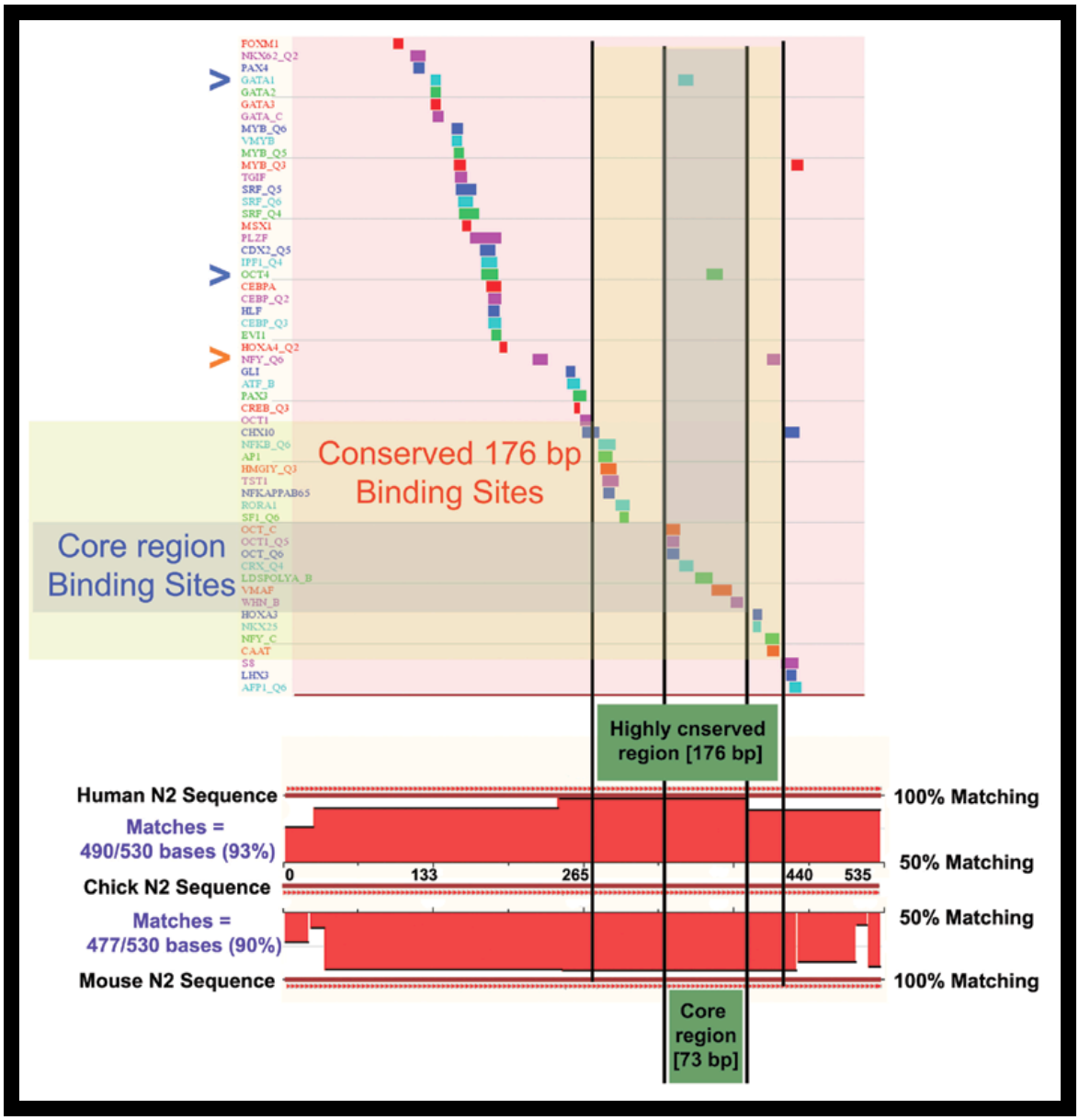

Figure Appx. 2: Sequence analysis of the N2 enhancer and localization of conserved Transcription Factors Binding Sites (TFBS).

This figures shows a summary of the N2 enhancer sequence analysis in the mouse and chick with location of the 176 bp and 73 bp core regions as well as the main TFBS location within its sequence. A total of 3 POU binding sites are present in the 176 bp sub region out of which two are embedded in the 73 bp core region.